IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No.	:	10/076,071	Confirmation No. 8825
Applicant	:	David Bar-Or	
Filed	:	February 13, 2002	
TC/A.U.	:	1653	
Examiner	:	Anand Desai	
Docket No.	:	4172-3-2	
Customer No.:		22442	

Commissioner for Patents
P.O. Box 1450
Alexandria VA 22313-1450

DECLARATION OF DR. DAVID BAR-OR

I, David Bar-Or, M.D., declare that:

1. I am the same David Bar-Or who is named as an inventor on the above-referenced patent application. I am employed as Chief Scientific Officer and Director Of Research by DMI BioSciences, Inc. ("DMI"), the assignee of the above-referenced patent application. I am also Chairman of the Board Of Directors of DMI.

2. Experiments in addition to those described in the above-referenced application were performed to confirm the ability of several metal-binding peptides of the invention to bind copper and to demonstrate the ability of these peptides to inhibit angiogenesis. The peptides that were tested varied by size of the peptide, sequence of the peptide (including different sizes and types of amino acids), hydrophobicity, hydrophilicity and other properties (see, for instance, Exhibit A, which contains a listing of the peptides). The thirty-two peptides were synthesized by standard techniques by the following companies: DMI Synthesis Ltd. (Cardiff, UK) (formerly Bowman Research Ltd.), Global Peptide (Ft. Collins, Colorado), Cambridge Research Biochemicals Ltd. (Cleveland, UK) and Diosynth.

3. Experiments were performed in my laboratory under my supervision to determine the ability of the peptides to inhibit the production of hydroxyl radicals caused by copper. The

experiments and the results of the experiments are described in Exhibit A, which is attached hereto and incorporated herein by reference. As can be seen from Exhibit A, most of the peptides gave complete inhibition of hydroxyl radical production at peptide:copper ratios of 4:1, and several of the peptides effectively inhibited hydroxyl radical production at peptide:copper ratios of 2:1 or less. These results show that the peptides inhibited a copper-mediated reaction and confirm the ability of the peptides to bind copper ions.

4. Additional experiments were performed in my laboratory under my supervision to determine the ability of the same peptides to inhibit the release of interleukin-8 (IL-8) from human umbilical vein endothelial cells (HUVECs). These additional experiments and the results of the experiments are described in Exhibit B, which is attached hereto and incorporated herein by reference. As can be seen from Exhibit B, all of the peptides inhibited IL-8 release from the HUVECs, and most of the peptides gave very high levels of inhibition of IL-8 release. IL-8 is a potent promoter of angiogenesis, and the results provide evidence that the metal-binding peptides of the invention have the ability to inhibit angiogenesis.

5. Further experiments were performed in my laboratory under my supervision to determine the ability of the peptides to inhibit the proliferation of HUVECs. These additional experiments and the results of the experiments are described in Exhibit C, which is attached hereto and incorporated herein by reference. As can be seen from Exhibit C, most of the peptides (27/32) inhibited HUVEC proliferation. Proliferation of endothelial cells is the first of the three stages of angiogenesis, and the results provide further evidence that the metal-binding peptides of the invention have the ability to inhibit angiogenesis.

6. The copper binding stability constants of eleven of the metal-binding peptides of the invention were determined by a third party contract research organization pursuant to a contract with DMI. These additional experiments and the results of the experiments are described in Exhibit D, which is attached hereto and incorporated herein by reference. As can be seen from Exhibit D, the eleven peptides had copper binding (stability) constants indicating that they bind copper well.

7. Nine of the peptides were tested in the chicken egg chorioallantoic membrane (CAM) assay for angiogenesis by a third party research organization pursuant to a contract with DMI. A

description of the experimental protocol is attached hereto as Exhibit E, which is incorporated herein in its entirety by reference. Data received from the research organization showed that all of the peptides caused inhibition of angiogenesis in this *in vivo* model of angiogenesis. The data are also summarized in Exhibit E. It was subsequently determined that the concentrations of the peptides used in this testing were substantially lower than the concentrations estimated to be needed to bind all of the copper present in the eggs. For instance, in the case of DAHK, it was estimated that at least 7X less than needed for best results was used. Thus, it is anticipated that higher levels of inhibition will be obtained when higher, more optimum concentrations of the peptides are used.

8. All statements made herein of my own knowledge are true, and all statements made on information and belief are believed to be true. I am aware that willful false statements and the like are punishable by fine, imprisonment or both (18 U.S.C. 1001) and may jeopardize the validity of this application or any patent issuing thereon.

David Bar-Or

David Bar-Or, M.D.

Date: April 20, 2006

Data Demonstrating That Additional Peptides Inhibit
The Generation Of Reactive Oxygen Species (ROS)

The ability of several peptides to inhibit the production of hydroxyl radicals was tested. The peptides that were tested are listed in Table A and B below. Those listed in Table A were obtained from Bowman Research UK Ltd., Newport, Wales, UK and those listed in Table B were obtained from either Cambridge Biotechnology Research, Cambridge, UK or Global Peptide, Fort Collins, Colorado. All peptides were composed of L-amino acids, unless indicated otherwise.

The ability of the peptides listed in Tables A and B to inhibit the generation of hydroxyl radicals was tested. Hydroxyl radicals were generated by mixing Cu(II) and ascorbic acid. When deoxyribose was added, the hydroxyl radicals, if present, attacked the deoxyribose to produce fragments. Heating the fragments at low pH produced malonaldehyde that, upon the addition of 2-thiobarbituric acid (TBA), yielded a pink chromogen which was measured spectrophotometrically at 532 nm. Thus, absorbance at 532 nm is a measure of the damage to deoxyribose and, therefore, of hydroxyl radical formation.

To perform the assay, (a) 50 μl of 200 μM $CuCl_2$ in water, (b) water or enough of one of the test peptides in water (total volume of 150 μl) to give peptide:copper ratios of 1:4, 1:2, 1:1, 2:1, 4:1, 6:1 and 8:1, and (c) 525 μl buffer (20 mM KH_2PO_4 buffer, pH 7.4) were added to test tubes. The test tubes were incubated for 15 minutes at room temperature. Then, 25 μl of 20 mM ascorbic acid in buffer and 250 μl of 7.5 mM 2-deoxy-D-ribose in water were added to each test tube, and the test tubes were incubated for 1 hour at 37°C. Finally, 1 ml of 1% (w/v) TBA in 50 mM NaOH and 1 ml of glacial acetic acid were added to each test tube, and the test tubes were incubated in boiling water for 15 minutes. After the test tubes had cooled for 15 minutes, the absorbance at 532 nm was read. The results are presented in the tables below and the attached Figures.

TABLE A

PEPTIDE	ABBREVIATION	FIGURE	RATIO*
Ala Ala His Lys	AAHK	A	4:1
Ala Ala His Ala	AAHA	B	4:1
Leu Gly His	LGH	D	4:1
Ser Gly His	SGH	E	2:1
Asn Gly His	NGH	F	4:1
Lys Gly His	KGH	G	4:1
Cys Gly His	CGH	H and V	8:1 (about 40%)
Arg Thr His	RTH	I	4:1
Ser Ser His	SSH	J	4:1
Thr Leu His	TLH	K	2:1
Asp Ala His Gly Gly	DAHGG	L	4:1
Asp Ala His Gly Gly Orn Ala His	DAHGGOAH	M	4:1
Asp Ala His Gly Met Thr Cys Ala Arg Cys	DAHGMTCARC	N	2:1
Asp Ala His Gly Met Thr Cys Ala Asn Cys	DAHGMTCANC	O	1:1
Asp Ala His Arg Arg Arg Arg Arg Arg	DAHRRRRRR	P and V	6:1
D-Ala D-Ala D-His D-His	D-AAHH	Q	4:1
D-Ser D-Ser D-His	D-SSH	R	4:1
D-Phe D-Gly D-His	D-FGH	S	4:1
D-Asp D-Ala D-His D-Lys	D-DAHK	T and Example 7	2:1

Asp Ala His Lys - NH_2 (the carboxyl of Lys is amidated)	DAHK-NH_2	U	2:1
Asp Ala His Lys	DAHK	Examples 7 and 10	2:1
Asp Ala His Lys Ser Glu Val Ala His Arg Phe Lys	DAHKSEVAHRFK	Example 10	2:1

*Unless otherwise noted, "Ratio" means the peptide:copper ratio where >95% inhibition was observed.

TABLE B

PEPTIDE	ABBREVIATION	FIGURE	RATIO*
Asp Ala His Gly Met Thr Cys Ala Asn Cys	DAHGMTCANC	AA	1:1
His Ala His Gly Met Thr Cys Ala Asn Cys	HAHGMTCANC	BB	2:1
Asp Ala His Lys Gly Met Thr Cys Ala Asn Cys	DAHKGMTCANC	CC	1:1
Asp Ala His Gly γGlu Cys Gly	DAHG-γE-CG	DD	2:1
D-Ala D-Ala D-His D-His	D-AAHH	EE	2:1
Arg Ala His Ala	RAHA	FF	2:1
Asp Ala His Lys Gly Gly His His	DAHKGGHH	GG	2:1
Ala His His Ala	AHHA	II	4:1
His Ala His His	HAHH	JJ	4:1 (74% inhibition)
His Ala His Ala	HAHA	KK	2:1 (85% inhibition)
Ala Ala His His	AAHH	LL	2:1

*Unless otherwise noted, "Ratio" means the peptide:copper ratio where >95% inhibition was observed.

As can be seen from the figures, most of the peptides gave essentially complete inhibition (greater than 95%) of hydroxyl radical production at peptide:copper ratios of 4:1. However, several peptides effectively inhibited hydroxyl radical production at peptide:copper ratios of 2:1 or less.

Figure A

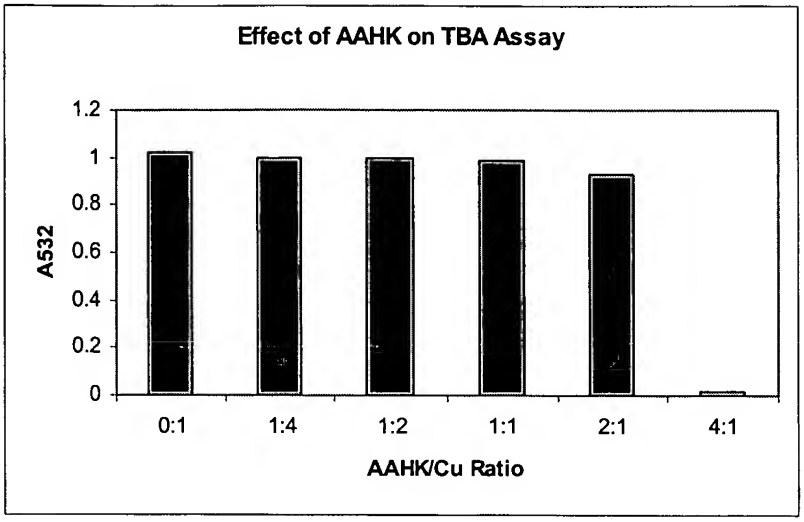


Figure B

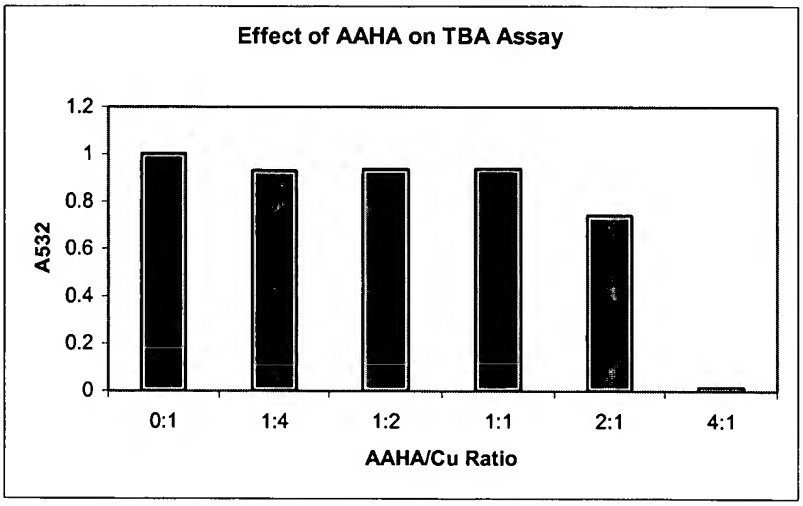
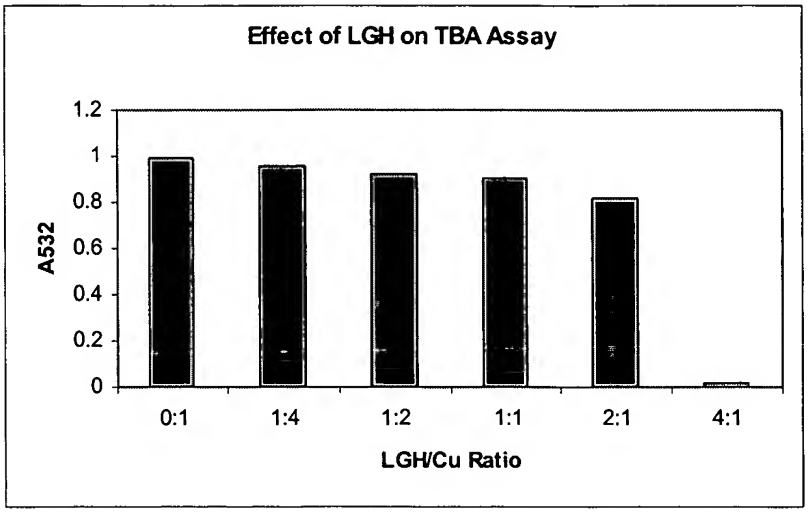


Figure D


Effect of LGH on TBA Assay
A532
1.2
1
0.8
0.6
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0.2
0
0:1
1:4
1:2
1:1
2:1
4:1
LGH/Cu Ratio

Figure E

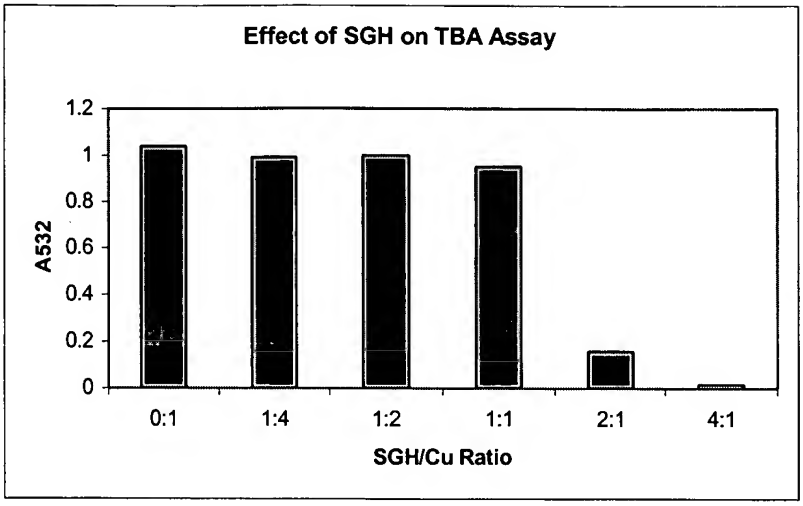


Figure F

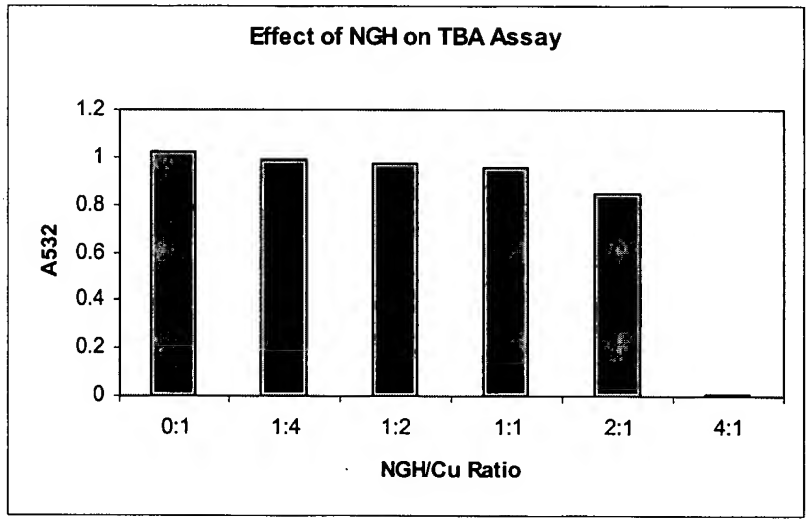


Figure G

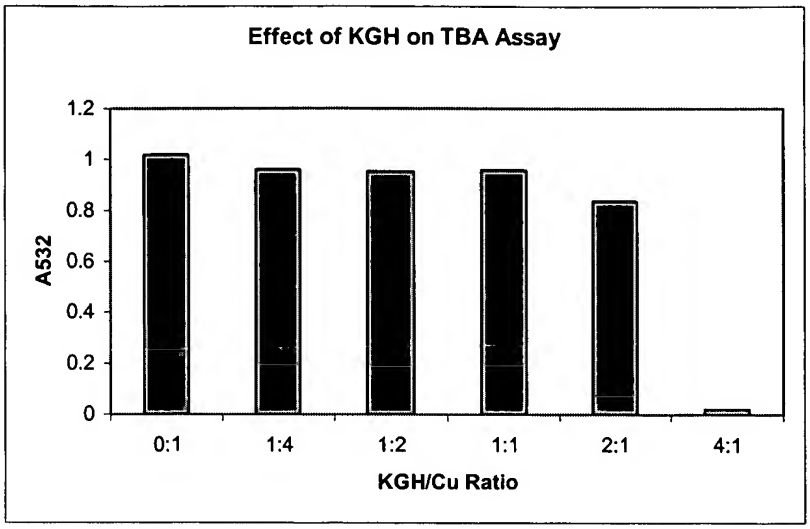


Figure H

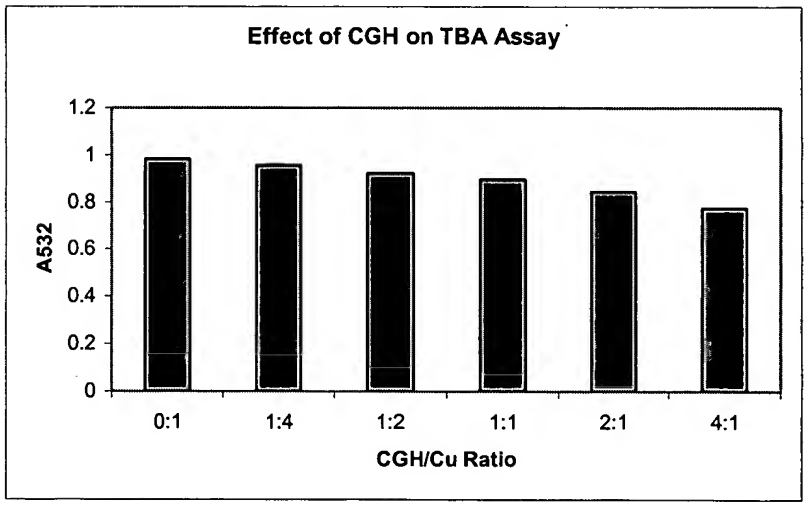


Figure I

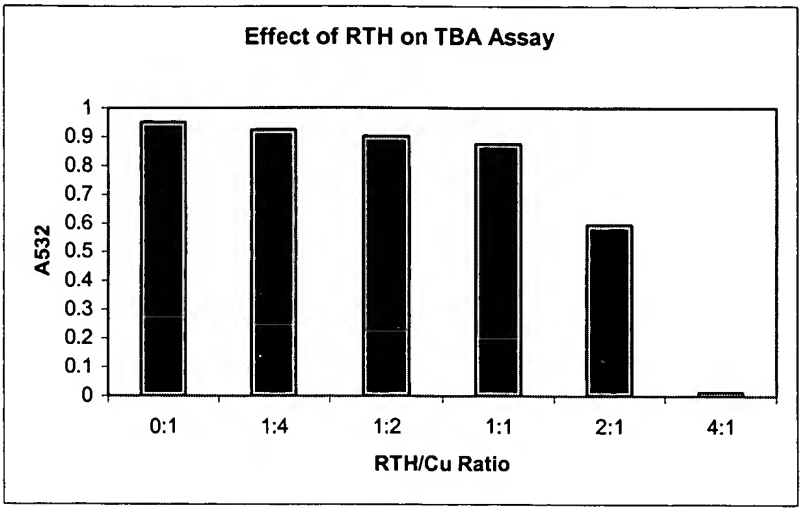


Figure J

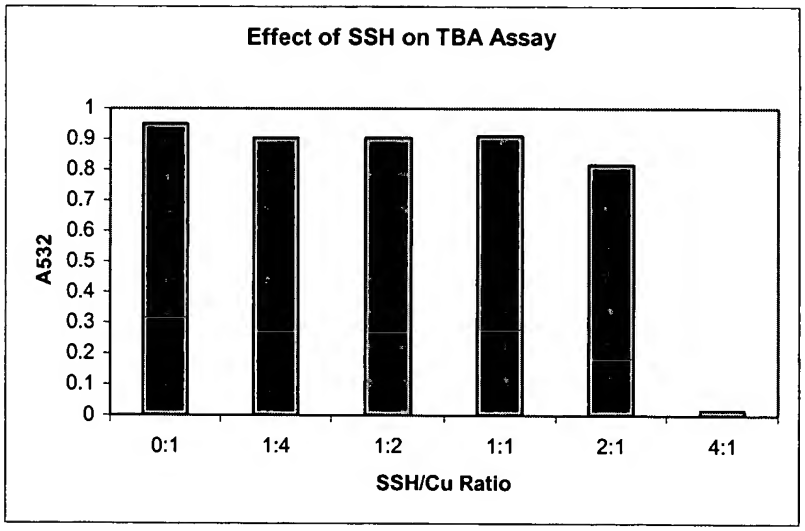
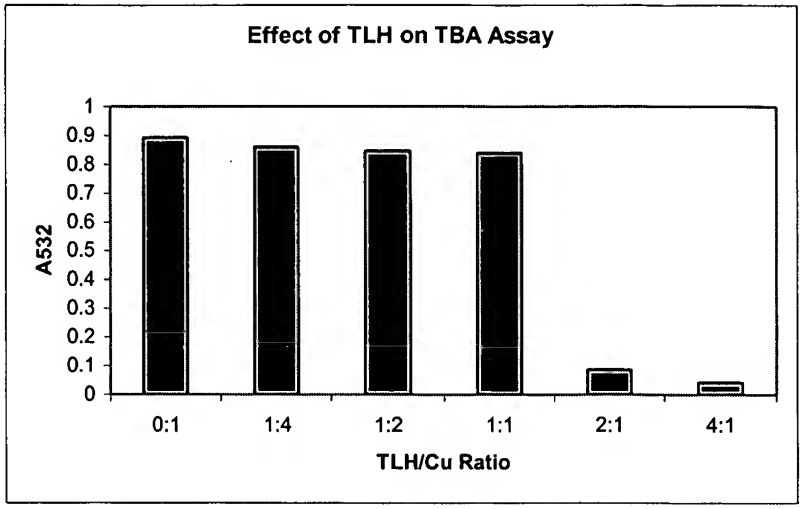


Figure K



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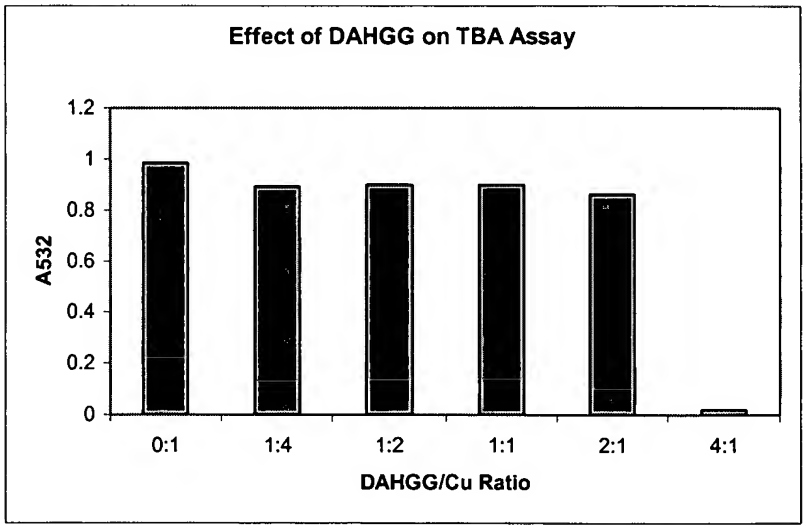

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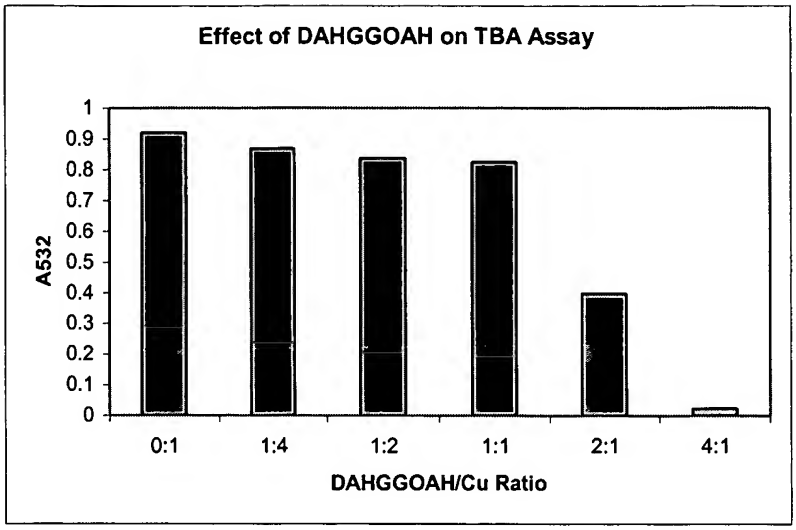

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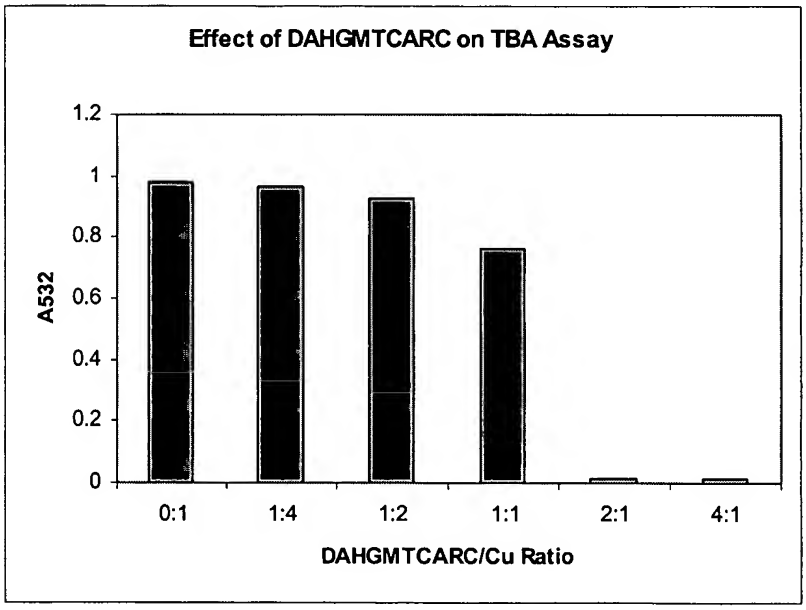


Figure O

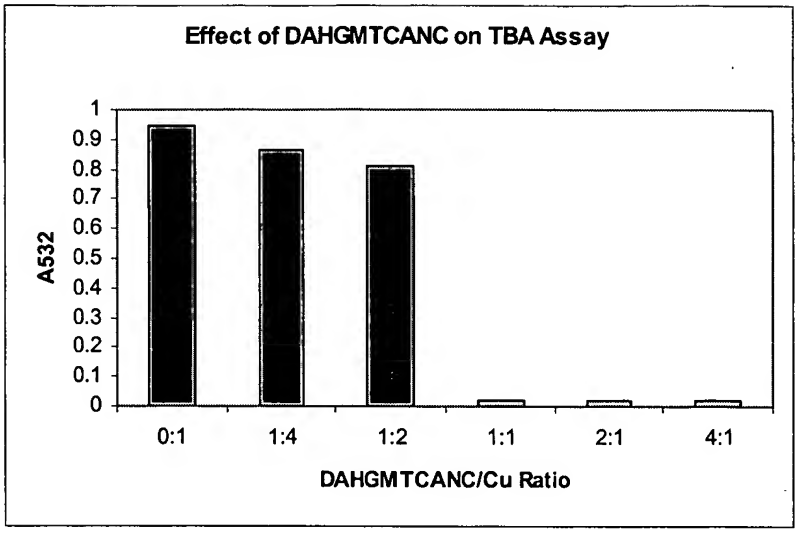


Figure P

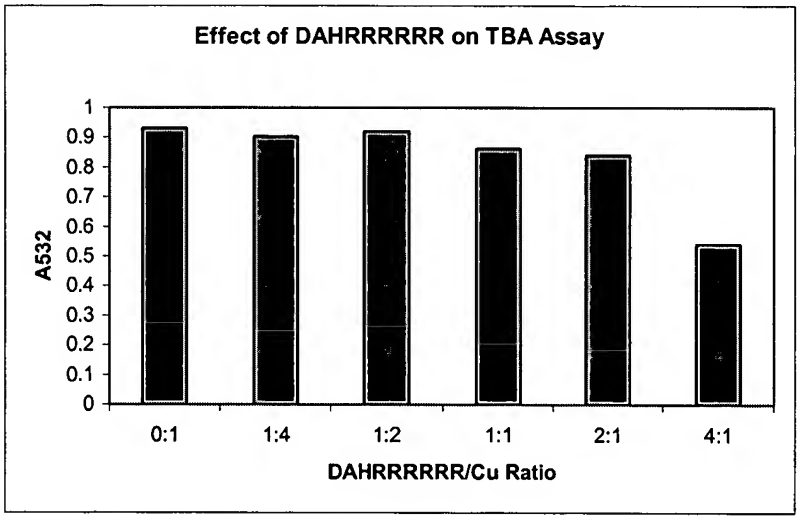


Figure Q

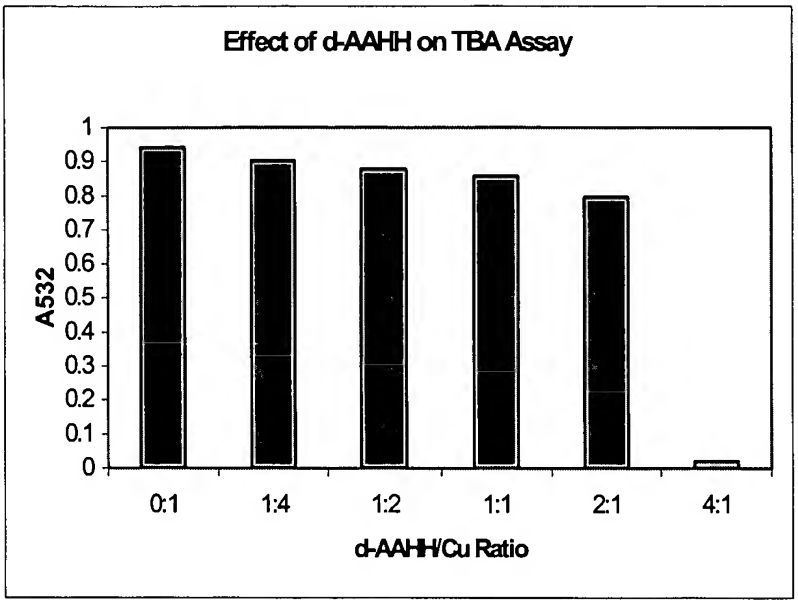


Figure R

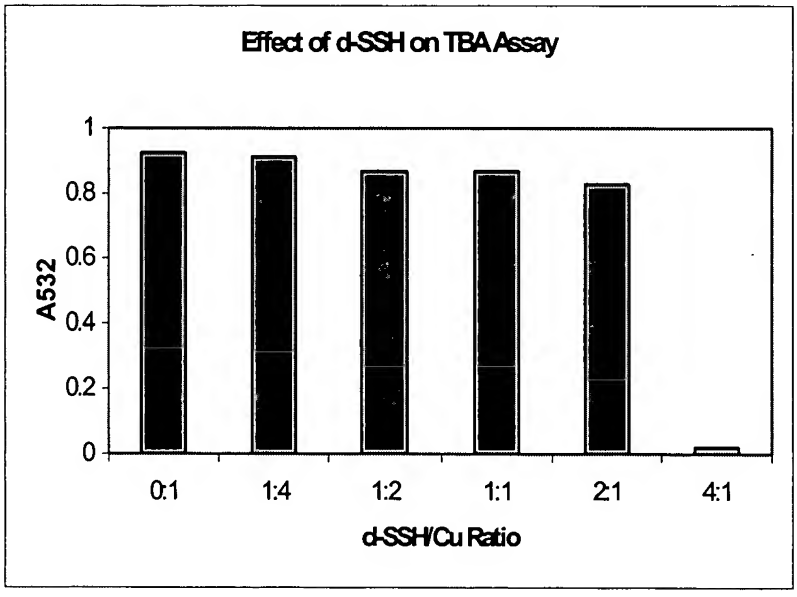


Figure S

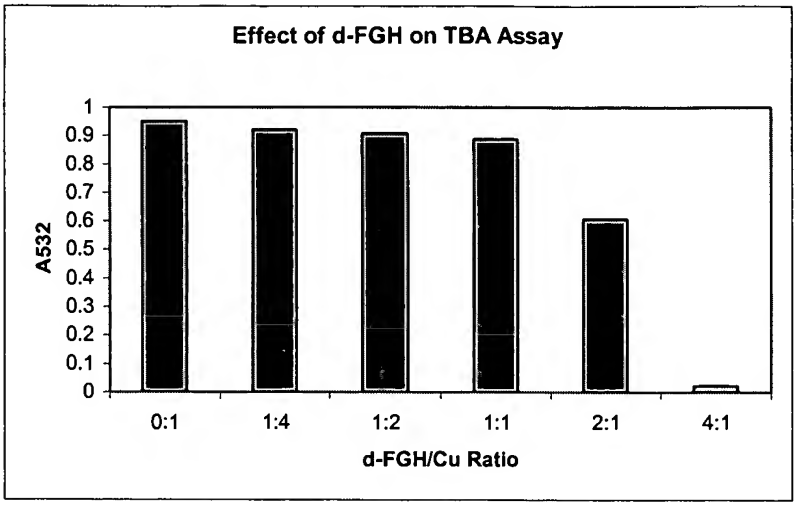


Figure T

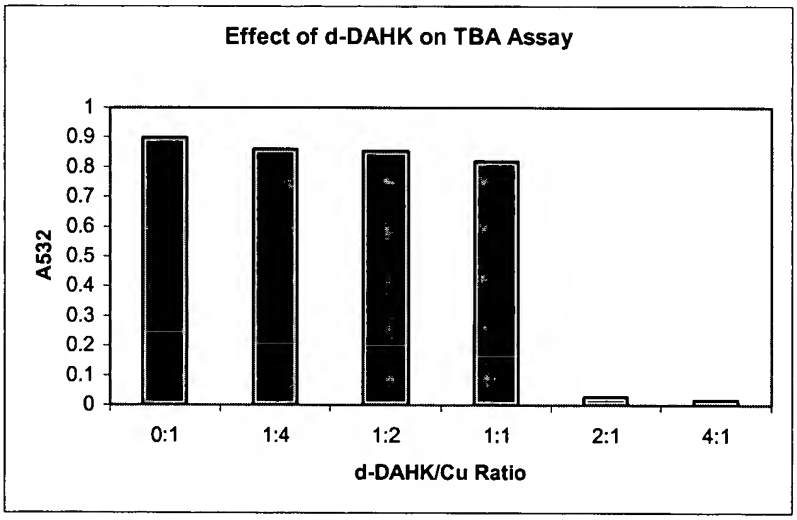


FIGURE U

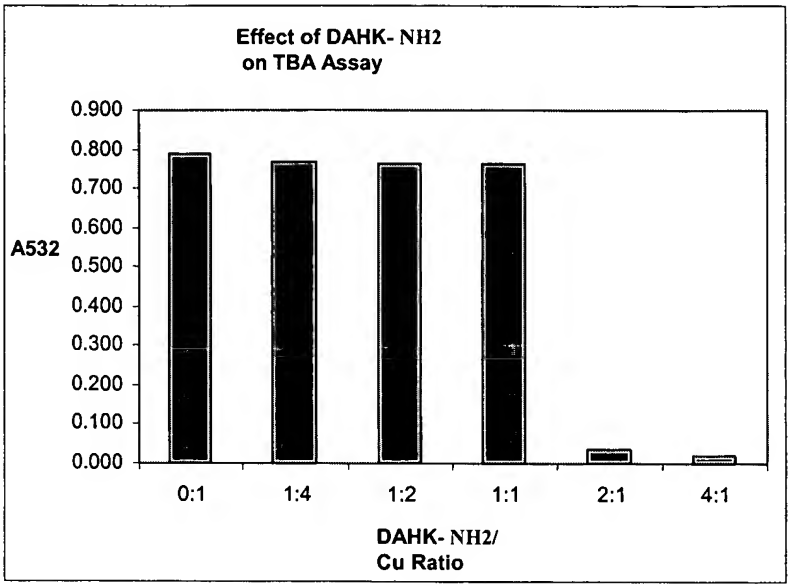


FIGURE V

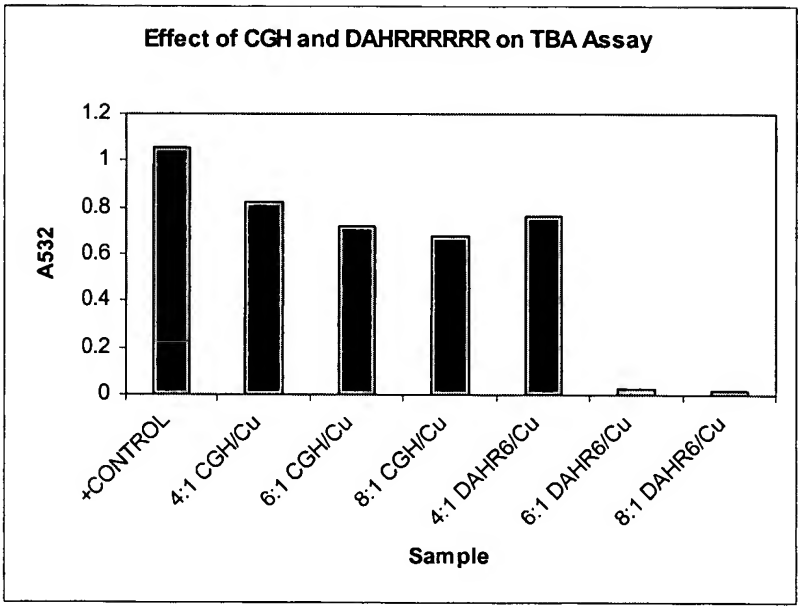


FIGURE AA

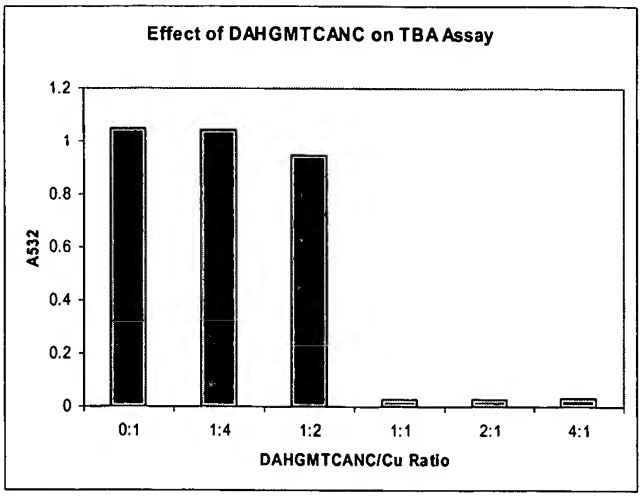


FIGURE BB

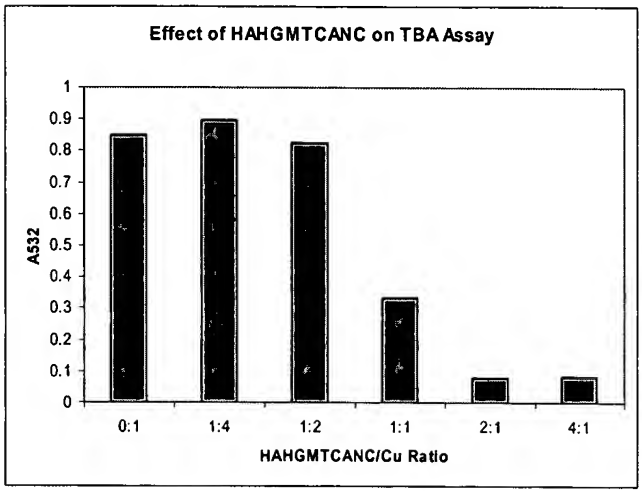


FIGURE CC

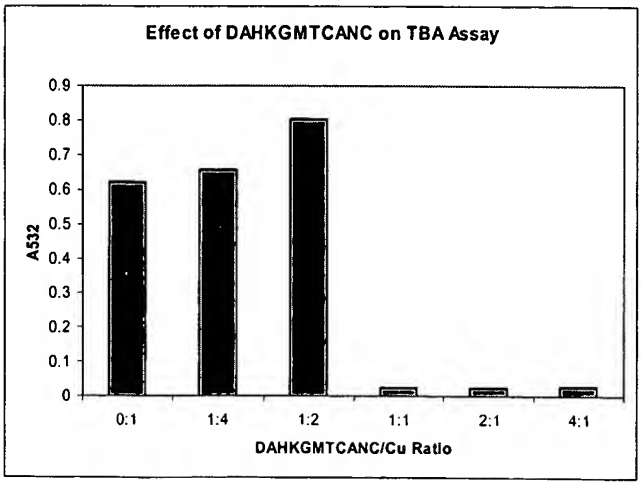


FIGURE DD

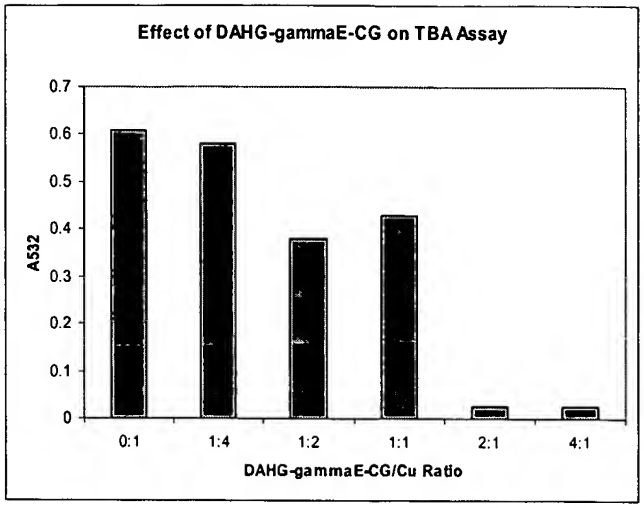


FIGURE EE

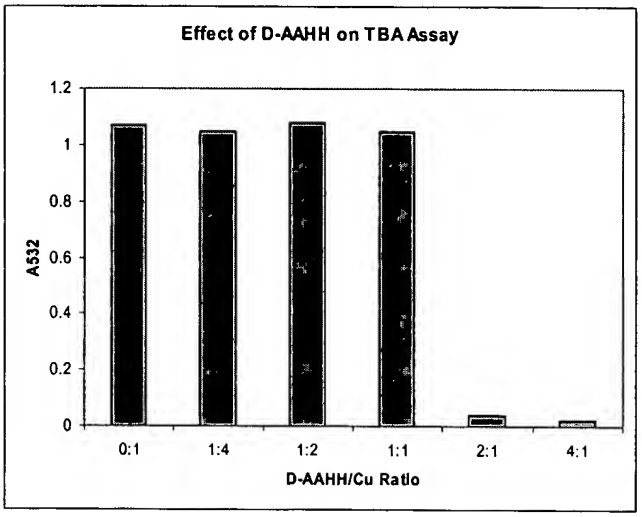


FIGURE FF

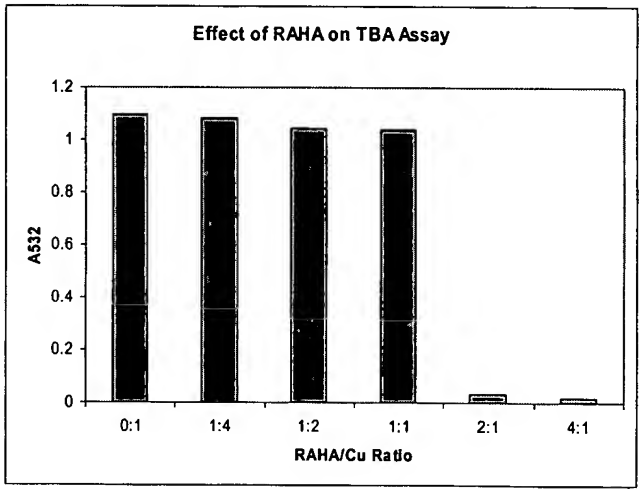


FIGURE GG

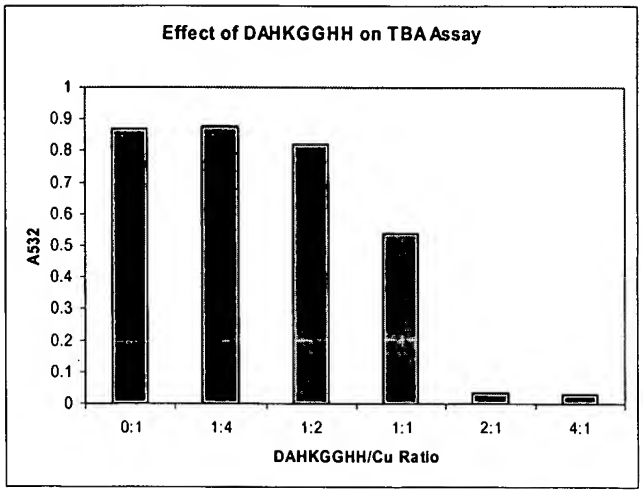


FIGURE HH

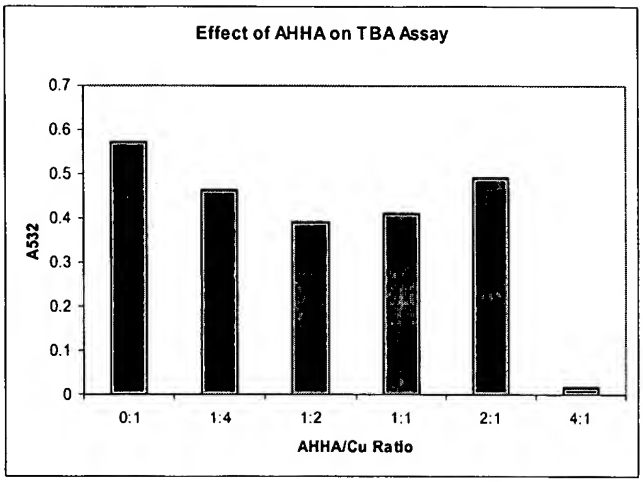


FIGURE II

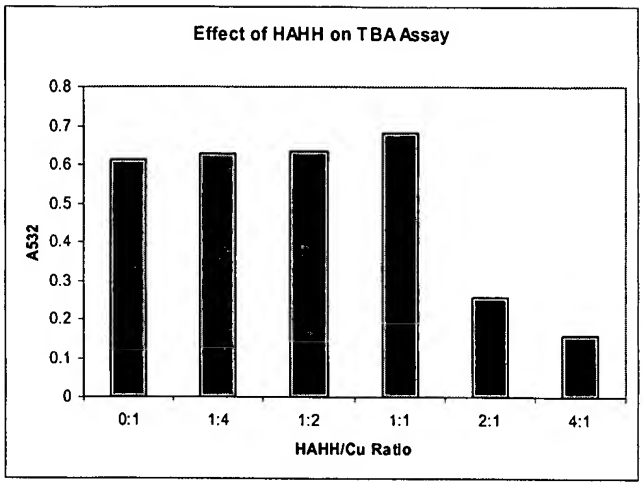


FIGURE JJ

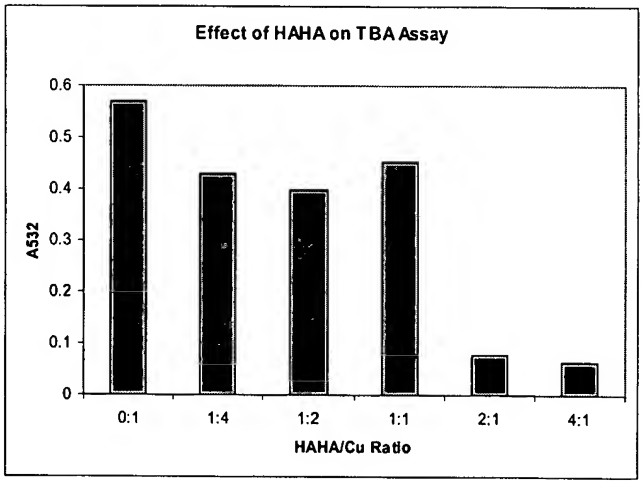
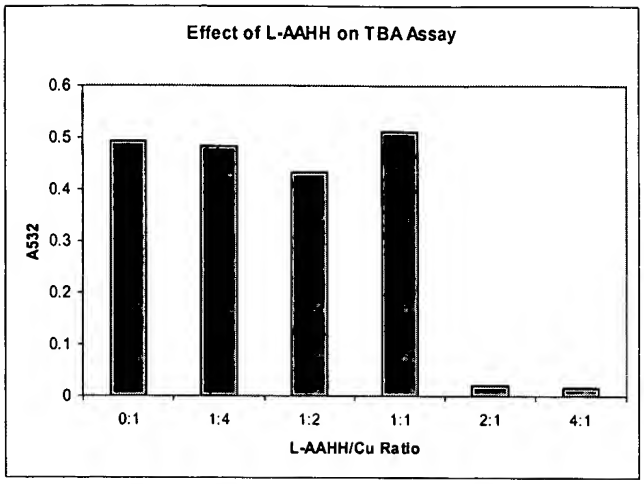


FIGURE KK


Effect of L-AAHH on TBA Assay
A532
0.6
0.5
0.4
0.3
0.2
0.1
0
0:1
1:4
1:2
1:1
2:1
4:1
L-AAHH/Cu Ratio

Inhibition Of IL-8 Release

Interleukin 8 (IL-8) is a pro-inflammatory cytokine and a potent chemoattractant and activator of neutrophils. It has also been reported to be a chemoattractant and activator of T-lymphocytes and eosinophils. IL-8 is also a potent pro-angiogenic factor.

The release of IL-8 by human umbilical vein endothelial cells (HUVECs) was investigated. To do so, HUVECs, human source lot number 9713 (Cambrex, passage 4 through 8) were plated on 48-well plates in endothelial growth medium-2 (EGM-2) containing 2% fetal calf serum (FCS), hydrocortisone, human fibroblast growth factor B, vascular endothelial growth factor, recombinant insulin-like growth factor-1, ascorbate, human epithelial growth factor, gentamycin and heparin (all obtained from Cambrex) and allowed to grow to >90% confluency at 37°C, 5% CO_2. Prior to addition of peptides, all wells were rinsed once with EGM-2 medium which did not contain ascorbate and FCS, but did contain all the growth factors and was supplemented with 1X ITSS (insulin, transferrin and sodium selenite) (obtained from Sigma as 100X stock solution which is diluted with EGM-2 to give 1X).

The cells were treated as follows with all concentrations listed as final concentrations:

- -CON well = 50 nM phorbol 12-myristate 13-acetate (PMA) (obtained from Sigma)
- +CON well = 50 nM PMA + 25 μM $CuCl_2$
- 2:1 peptide/Cu wells = 50 nM PMA + 50 μM peptide + 25 μM $CuCl_2$
- 4:1 peptide/Cu wells = 50 nM PMA + 100 μM peptide + 25 μM $CuCl_2$

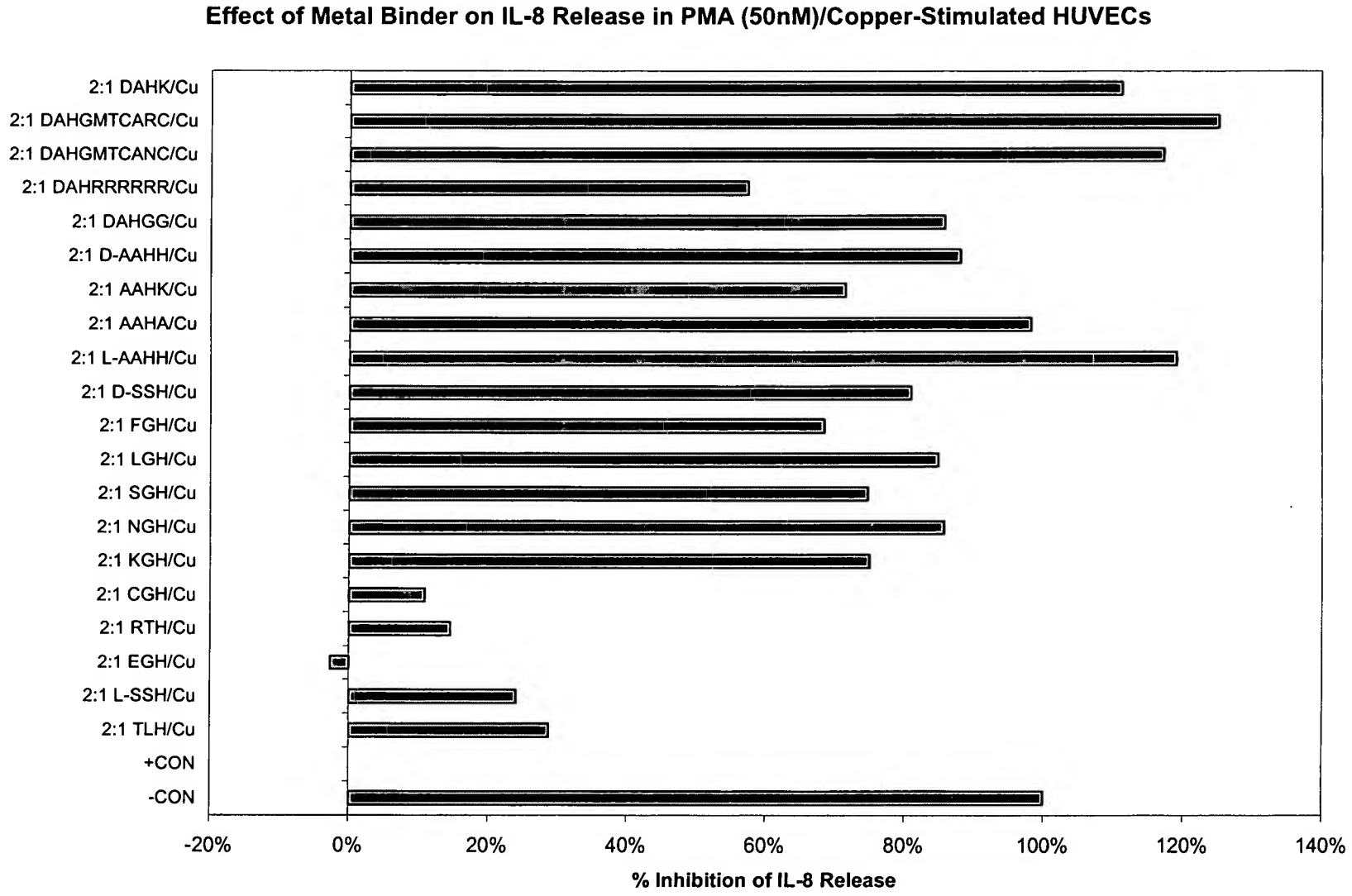
All additives were made up in EGM-2 medium (that did not contain ascorbate or FCS, but was supplemented with 1X ITSS). Total volume in each well was 500 μL per well. After the additives were added, the cells were incubated for 24 hours at 37°C, 5% CO_2. After the 24-hour incubation, 400 μL of each supernatant were collected.

The concentration of IL-8 in each supernatant was determined by an IL-8 ELISA assay performed in duplicate on each collected supernatant. To perform the ELISA, matched pairs of antibodies against human IL-8 were purchased from Pierce Biotechnology. ELISA strip well plates were coated with 1 μg/ml of antibody to IL-8 (in phosphate-buffered saline (PBS)) overnight at room temperature. The plates were then blocked using a 4% BSA solution in PBS

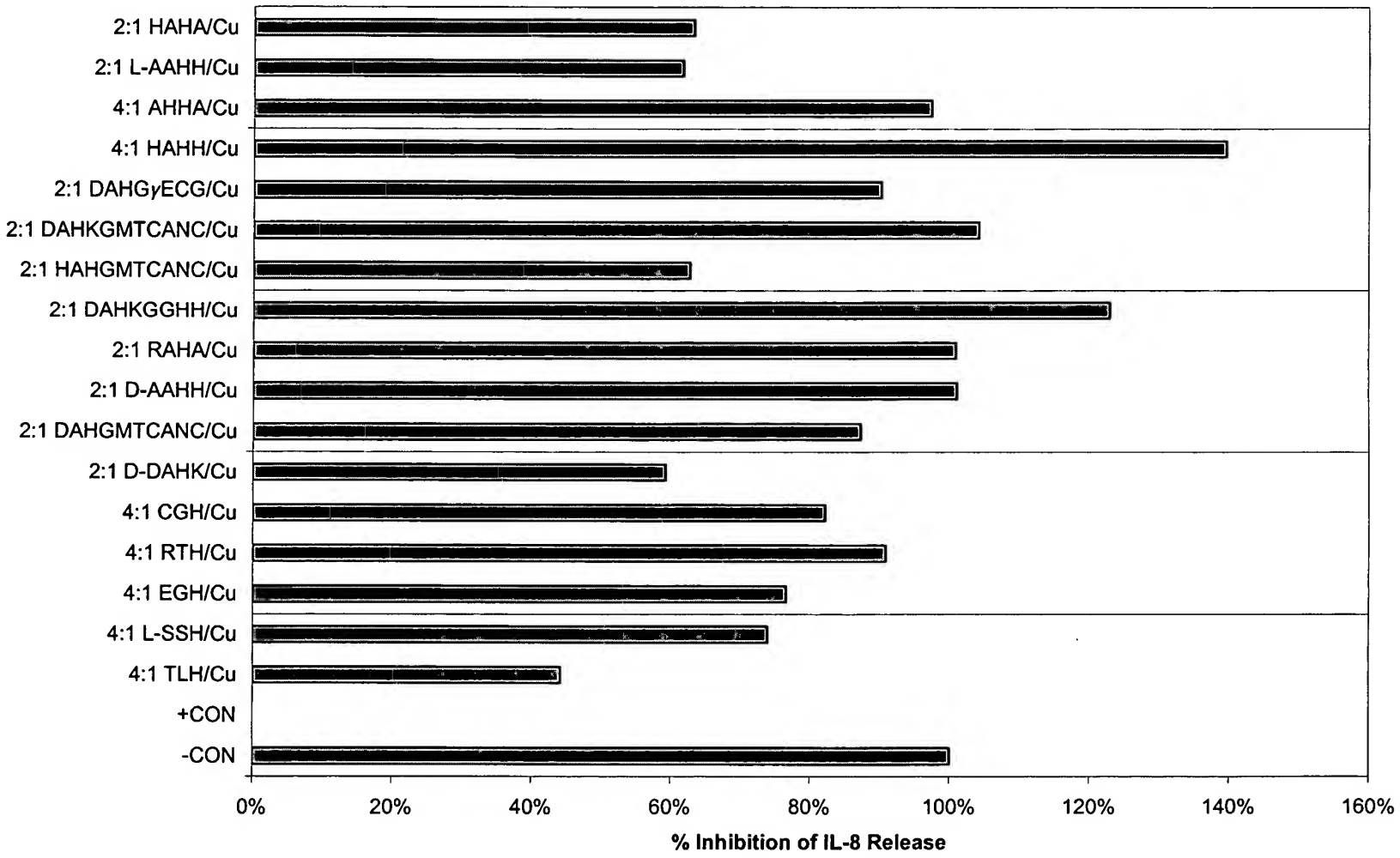
for one hour, washed twice with 50 mM Tris, pH 8.0, containing 0.1% Tween 20, followed by the addition of 50 µl of a sample supernatant or IL-8 standard per well in duplicate. The plates were incubated at room temperature for one hour and then washed using 50 mM Tris, pH 8.0, with 0.1% Tween 20. Then, solutions of 100 ng/ml biotinylated second antibody to IL-8 were made in blocking buffer, and 100 µl were added per well. The plates were incubated for 1 hour and washed again. A 1:12,000 dilution of Strepavidin HRP (Pierce Biotechnology) conjugate was made in blocking buffer, and 100 µl were added to the wells and incubation continued for 30 minutes. A final wash step was performed, after which 100 µl Pierce Biotechnology TMB substrate were added to each well. Color was developed for 5-10 minutes and stopped by adding 100 µl 0.18 N H_2SO_4. OD was determined using a microplate reader with a 450 nm filter and a 530 nm background subtraction.

The results are shown in the attached figures. The percent inhibition was calculated by assigning the negative control (-CON) a value of 100% inhibition and the sample containing 25 µM $CuCl_2$ only (+CON) a value of 0% inhibition. The wells containing the peptides were compared to these two and assigned % inhibition using the following equation:

$$\% \text{ Inhibition} = 1 - \frac{[\text{IL-8 release (ng/ml) in peptide well}] - [\text{IL-8 release (ng/ml) in -CON}]}{[\text{IL-8 release (ng/ml) in +CON}] - [\text{IL-8 release (ng/ml) in -CON}]} \times 100\%.$$


Effect of Metal Binder on IL-8 Release in PMA (50nM)/Copper-Stimulated HUVECs
2:1 DAHK/Cu
2:1 DAHGMTCARC/Cu
2:1 DAHGMTCANC/Cu
2:1 DAHRRRRRR/Cu
2:1 DAHGG/Cu
2:1 D-AAHH/Cu
2:1 AAHK/Cu
2:1 AAHA/Cu
2:1 L-AAHH/Cu
2:1 D-SSH/Cu
2:1 FGH/Cu
2:1 LGH/Cu
2:1 SGH/Cu
2:1 NGH/Cu
2:1 KGH/Cu
2:1 CGH/Cu
2:1 RTH/Cu
2:1 EGH/Cu
2:1 L-SSH/Cu
2:1 TLH/Cu
+CON
-CON
-20%
0%
20%
40%
60%
80%
100%
120%
140%
% Inhibition of IL-8 Release

Effect of Metal Binders on IL-8 Release in PMA (50nM)/Copper-Stimulated HUVECs



HUVEC Proliferation Assay

DMI BioSciences Inc. 2006

Materials:

- Human umbilical vein endothelial cells (HUVEC, human source lot number 9713; from Cambrex)
- Endothelial growth medium-2 (EGM-2; from Cambrex)
- Supplements for EGM-2 complete medium (2% FCS, hydrocortisone, human fibroblast growth factor B, VEGF, recombinant insulin-like growth factor-1, ascorbate, human epithelial growth factor, gentamycin and heparin) (obtained from Cambrex)
- Endothelial basal medium-2 (EBM-2; from Cambrex)
- 500X ITSS (insulin, transferrin and sodium selenite; from Cambrex)
- Celltiter Aqueous One Solution Cell Proliferation assay (Promega)
- Collagen IV from human placenta (Sigma); 1 mg/ml in acetic acid
- PI3 kinase inhibitor LY 294002 (Sigma); 10 mM in DMSO
- 2 mM and 4 mM stocks of test compounds; sterile filtered; in water
- 96 well culture plates and 75 cm^2 culture flasks
- Eppendorf repeater pipette and sterile combi-syringes
- Sterile filtered HPLC grade water
- Trypsin/EDTA and Trypsin neutralizing solution (Cambrex)
- FL 600 Microplate reader (BIO-Tek)

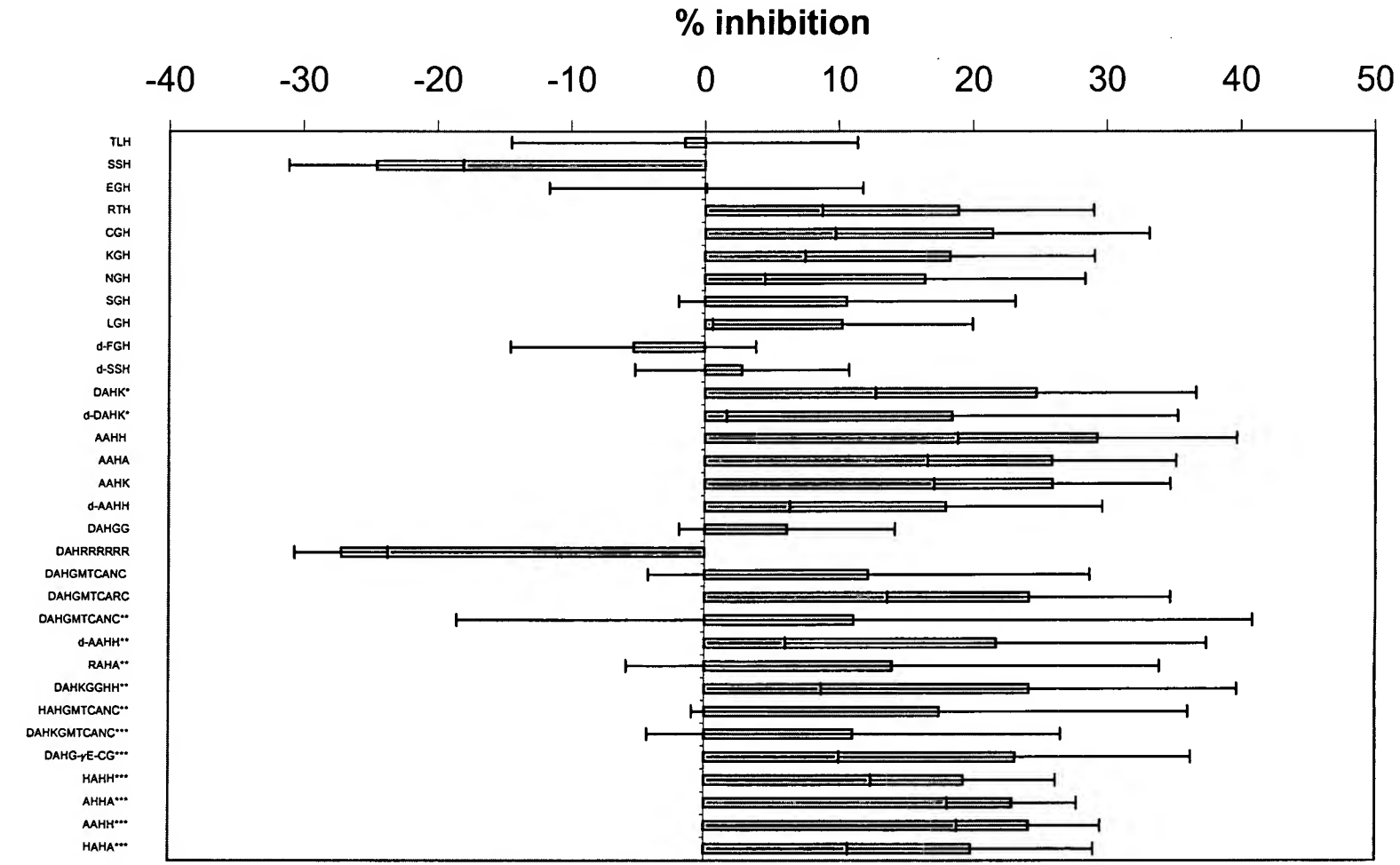
Protocol:

1. Passage 7 HUVEC 9713 were cultured in 75 cm^2 flasks using EGM-2 complete medium at 37°C and 5% CO_2.
2. When 60% confluence was obtained, the cells were detached from the plastic using Trypsin/EDTA and then placed in EBM-2 containing no culture supplements.
3. 800 μM solutions of test compounds in EBM-2 were prepared using sterile stocks in water, as well as control solutions containing equivalent amount of sterile water alone in EBM-2.
4. Growth factor preparation was made by adding 4 times the concentration of EGM-2 complete medium supplements to EBM-2 (excluding serum and ascorbic acid) plus 4 μg/ml collagen and 4X ITSS.
5. Background solution was also prepared by making a stock of 4 μg/ml collagen and 4X ITSS.
6. LY 294002 at a final concentration of 10 μM was used as the positive control.
7. 50 μl EBM-2, 50 μl growth factor preparation, and 50 μl of 800 μM test compound solutions or control solutions were then added to wells of a 96 well culture plate and the plates were incubated for 1 hour. All reactions were performed in triplicate.

8. 50 μl cell solution was added to each well to reach a seeding of 10,000 cells per cm^2 and the plates were placed back in the incubator. Final concentration of test compounds was 200 μM.
9. After 48 hours, 30 μl Promega cell titer solution was added to each well and the plates were incubated for an additional 4 hours.
10. OD at 530 nm was determined for each well in microplate reader with the average reading of three wells containing no cells subtracted.
11. Data presented as % inhibition or ((OD Growth factors-OD collagen only background)-(OD Test compound-OD collagen only background)/(OD Growth factors-OD collagen only background)) X 100. The % inhibition for the negative control (water) was subtracted from the % inhibition for each of the test compounds, and the results are shown in the attached Figure.

Serum free HUVEC proliferation % inhibition data

All peptides manufactured by Bowman, except * = Diosynth, ** = Cambridge, and * = Global Peptide**



Determination of Binding (stability) and Protonation Constants for Peptides

The binding (with Cu^{II} ions) and hydrogen ion constants for a number of peptides were determined. The method that was used for these determinations is described below.

1. Instrumentation:

The determinations of the stability constant were performed by using an automatic Potentiometric Titrator Model TIM 840, supplied by Radiometer Analytical Ltd equipped with a GLEE HyperQuad 2000 software programme capable of deconvoluting the titre values. The titrations were performed in a reaction vessel (100 mL) equipped with a calibrated thermometer (0-60° C),a pH micro electrode (Silver-Silver Chloride) and a Nitrogen inlet/outlet. The temperature of the titration vessel is maintained at 25 °C throughout the experimental procedure by a constant temperature water re circulator (Haake Model BC10).

2. Chemicals and ancillary equipments

1. Volumetric Standard Sodium Hydroxide solution (0.1M; Aldrich Lot No: 10508 JC)
2. Nitric Acid standard solution (0.1M)
3. Sodium nitrate - hemi penta hydrate (Aldrich, Lot No: 011597).
4. two stage de-ionised water (conductivity grade water)
5. Analytical balance (four place)

3. Experimental Procedure

3.1 Preparation of standard solutions

a) Sodium hydroxide solution (0.1M) was diluted as required with conductivity water and used. A simple acid base titration was performed to obtain its true concentration.

b) Nitric acid solution (0.1M) was prepared by diluting concentrated Nitric Acid with the appropriate volume of deionoised water.

c) 0.05 M Sodium Nitrate (electrolyte used in conductometric titrations) solution was prepared by dissolving a known amount of Analar grade Sodium Nitrate hemipentahydrate in deionoised water.

3.2 Instrument calibration

The electrode was calibrated daily with standard pH buffers (4-7 and 10) prior to analyses and the instrument was calibrated by titrating a known volume of nitric acid solution in electrolyte (50 mL) with sodium hydroxide solution (0.1 mole or as appropriate) at 25°C. The titre values was used to calculate electrolyte potential (E^o) and hence, the alkali impurity level was determined.

3.3 Establishing the minimum amount of ligand (peptide) required for analyzing peptides

In view of the limitation of the amount of peptide available for the determination of the Stability constants for the supplied peptides, experiment were performed to obtain protonation constant for Glycylglycine at two concentrations; 0.05 mmole and 0.025 mmole. Based on these results the minimum amount of test compound required for each of the analyses was determined to be 0.025mmole.

3.4 General method for the measurement of Protonation constants

The test compound (peptide; 0.025mmole) was dissolved in a solution consisting of Sodium Nitrate solution (0.1M; 1mL), Dilute Nitric Acid (1mL, 1 M) and was diluted to 50 mL in a volumetric flask. The solution was transferred to the jacketed titration chamber kept at 25 °C (water circulator). The solution was allowed to equilibrate and the titration with standard sodium hydroxide solution (0.025mole) was allowed to progress. A typical experiment consisted of incremental addition of 50 µL aliquots of the base (dilute sodium hydroxide solution) and until the pH of the solution reached 11.

The soft ware was programmed to collect electrode potentials at 3 second intervals until nine successive measurements agreed within ±0.1mV. From a plot of the pH and the neutralising degree (electrode potential) the acidity constant was determined. From the pKa values, the number of potential metal binding sites was assessed.

3.5 Binding constants

The experimental conditions used for the determination of the metal binding constants was similar to the above (acidity constant experiment) except that a part of sodium nitrate was replaced by a molar equivalent solution of the divalent copper (typically as Copper Nitrate solution) with the ratio of Cu ions to peptide as 1:1.

4. Results

The technique used for the determination of protonation and stability constants by the titrimetric method has been described above. The results giving the protonation constants (column 3) and the calculated stability constants (column 4) are presented in tabular form. The stability constants are quoted as $\log_{10}$ values, as is conventional.

In a majority of cases the peptides formed a 1:1 square planar complex with Cu^{II} ions at physiological pH conditions. The study showed that at lower pH, the dominant complexation with the metal ion involved carboxylic group.

5. Additional Information

A number of methods are known for determining stability constants. Because protons and metal ions compete for the donor group of the peptide, complex formation is investigated most accurately by pH titration. Several factors such as the conditions of measurements to include, temperature, maintenance of inert atmosphere, calibration of the pH electrode, etc. affect the measurement of stability constants. For this reason, adequate precautions were taken to ensure that the instrument and its accessories were properly maintained. An inert atmosphere was maintained throughout the experimental procedure and the electrolyte was chosen such that it did not react adversely with the metal ions. The temperature of the reaction vessel was maintained at a constant temperature (25°C) and the water was doubly distilled and deionised prior to use.

6. Calculation of stability constants

The data were processed by the program Glee and Hyperquad 2000, specifically designed for the calculation and refinement of electrode potential, Protonation and binding constants. The number of experimental data required by the software is 50, we applied between 75 – 100 data collected between pH 2.6 and pH 11 in order to obtain reliable constants.

For peptide P-12, we were unable to model the Protonation data in order to obtain stability constants. This was due to the model for the metal-ligand interaction proposed (and entered into the program) not matching the calculated results, i.e. does not account for the interaction behaviour that occurs in solution. In both cases, the models that apply successfully to all the other samples (i.e. a metal-peptide ratio of 1:1) were used as the starting point and failed. Alternative models were tried and also failed. The models attempted were: model 1: Cu-L; model 2: Cu-L, $Cu\text{-}L_{H\text{-}1}$; model 3: Cu-L, $Cu\text{-}L_{H\text{-}1}$, $Cu\text{-}L_{H\text{-}2}$; model 4: Cu-L, $Cu_2\text{-}L$, $Cu_2\text{-}L_2$, $Cu\text{-}L_2$; model 5: Cu-L, $Cu\text{-}L_{H\text{-}1}$, $Cu_2\text{-}L$, $Cu\text{-}L_2$. (note $Cu\text{-}L_{H\text{-}1}$ indicates a 1:1 Cu-peptide ratio but with a monodeprotonated peptide, $Cu\text{-}L_{H\text{-}2}$; means with a doubly deprotonated peptide). The P-12 sample was repeated twice, the same results were obtained each time.

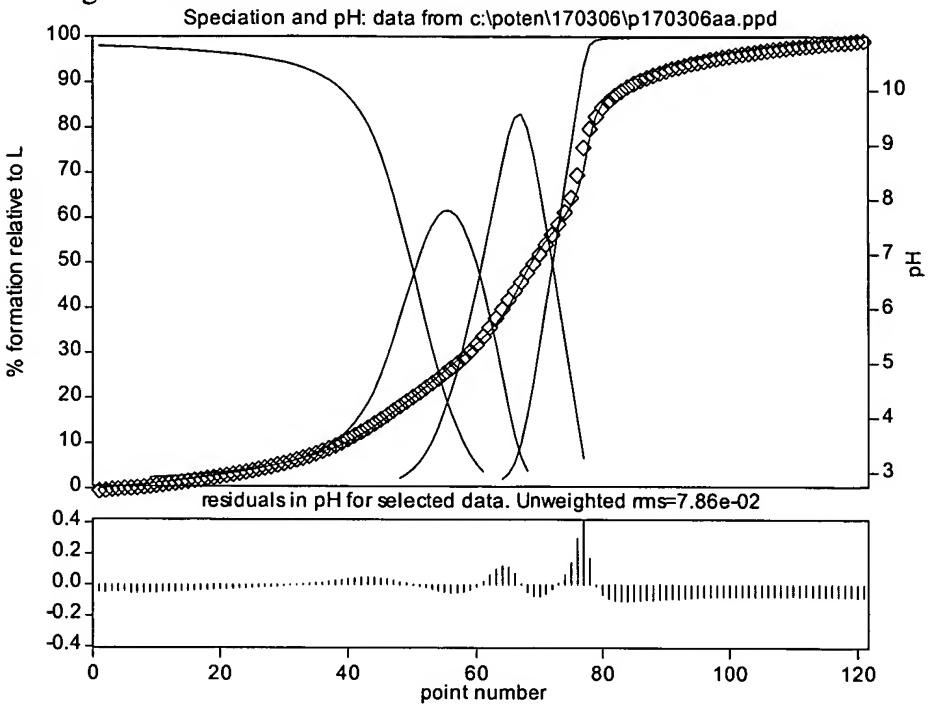
Reference No	Peptide	Protonation constants	Binding constants	Standard Deviation (σ)
P-1	L-DAHK	LH = 7.4039 LH_2 = 5.4090 LH_3 = 4.3808	Cu-P1 7.45	σ = 0.08
P-2	L-AAHH	LH = 7.1032	Cu-P2 5.30	σ = 0.03
P-3	D-DAHK	LH = 7.4912 LH_2 = 5.5693 LH_3 = 4.2777	Cu-P3 7.36	σ = 0.06
P-4	D-AAHH	LH = 7.8485 LH_2 = 6.4753	Cu-P4 8.38	σ = 0.03
P-5	L-DAHGMTCANC	LH = 9.2365 LH_2 = 7.9301 LH_3 = 6.5890 LH_4 = 3.2625 LH_5 = 2.2696	Cu-P5 13.15	σ = 0.06
P-7	L-HAHH	LH = 7.2219 LH_2 = 5.8754	Cu-P7 8.12	σ = 0.06
P-8	L-HAHA	LH = 7.4794 LH_2 = 5.6123	Cu-P8 8.04	σ = 0.05
P-9	L-RAHA	LH = 7.1226 LH_2 = 6.2678	Cu-P9 7.97	σ = 0.08
P-10	L-AHHA	LH = 7.6543 LH_2 = 6.0698	Cu-P10 9.80	σ = 0.02
P-11	L-Asp-L-Ala-L-His-L-Gly-L-γGlu-L-Cys-L-Gly	LH = 10.7658 LH_2 = 7.5954 LH_3 = 3.1836	Cu-P11 11.56	σ = 0.11
P-12	L-DAHKGGHH	LH = 7.4389 LH_2 = 6.1516	Cu-P12*	σ = 0.06

NOTES: Protonation constants relate to the types of protons that are removed during the titration

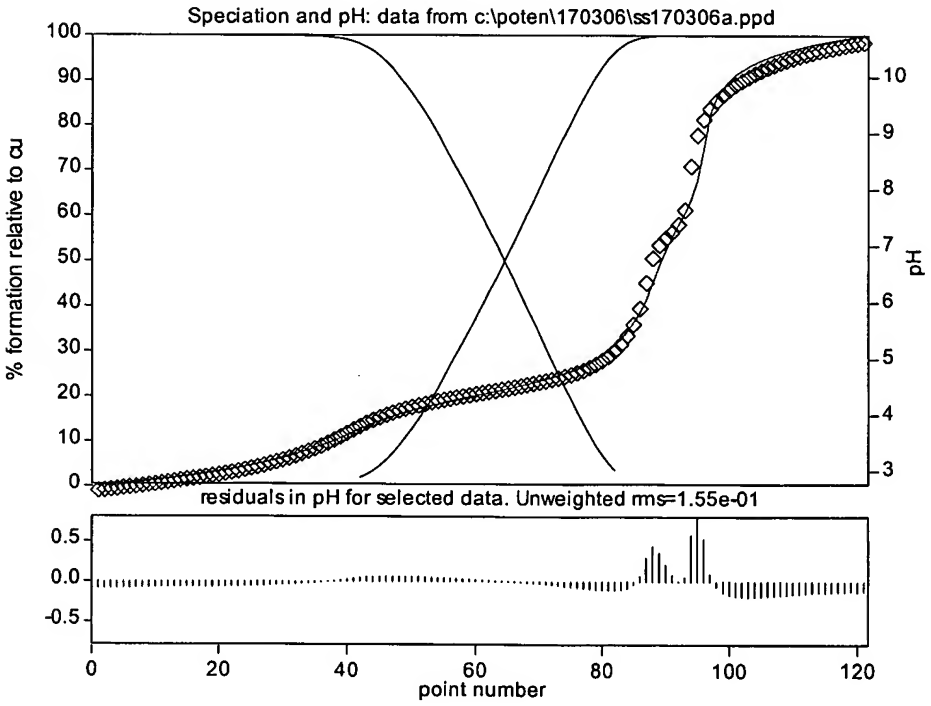
* Stability constant could not be estimated – see above.

7. Appendix 1 – Protonation and stability constant data and curve fits

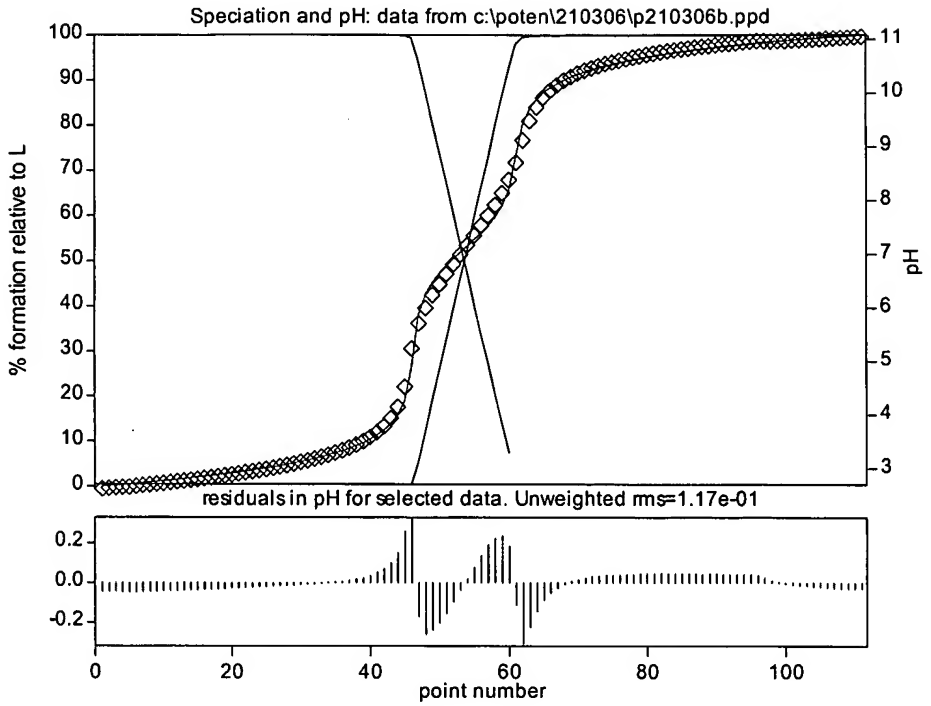
P-1 Ligand Protonation Constant



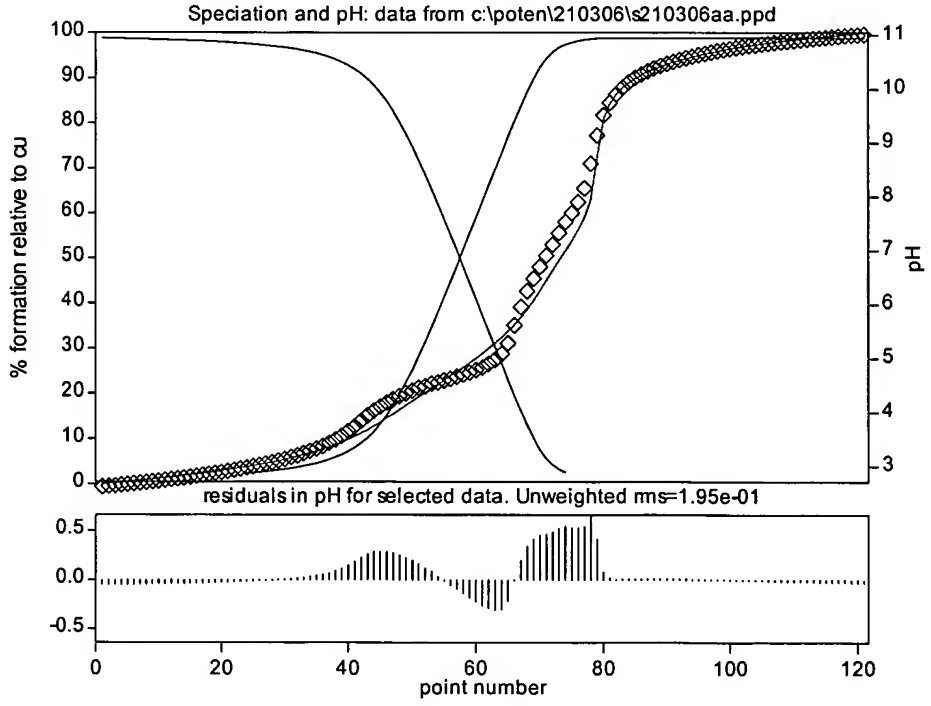
P-1 Metal Complex Stability Constant



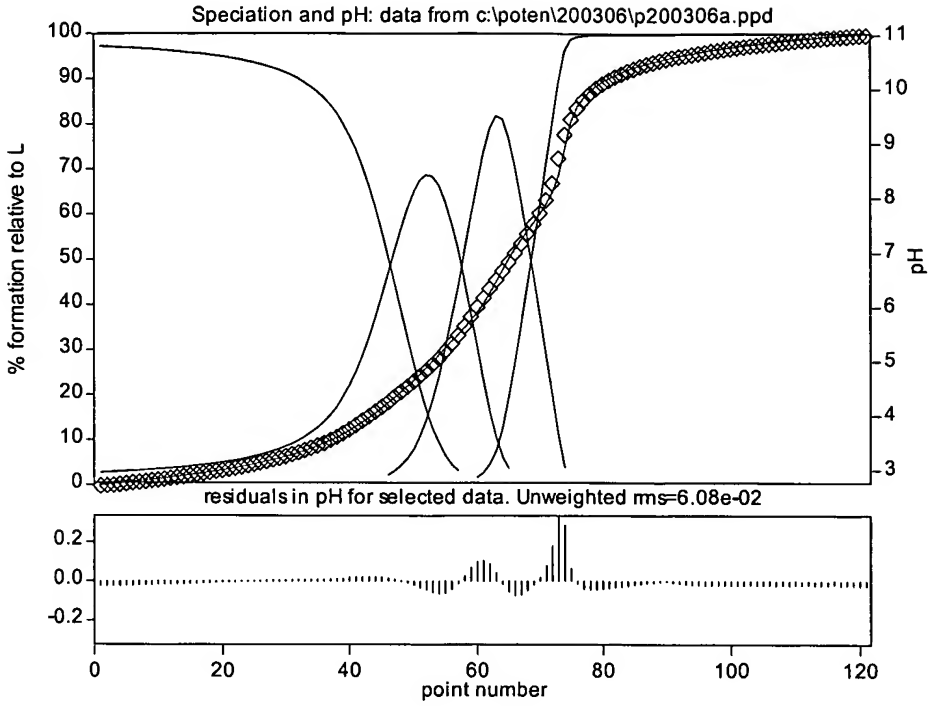
P-2 Ligand Protonation Constant



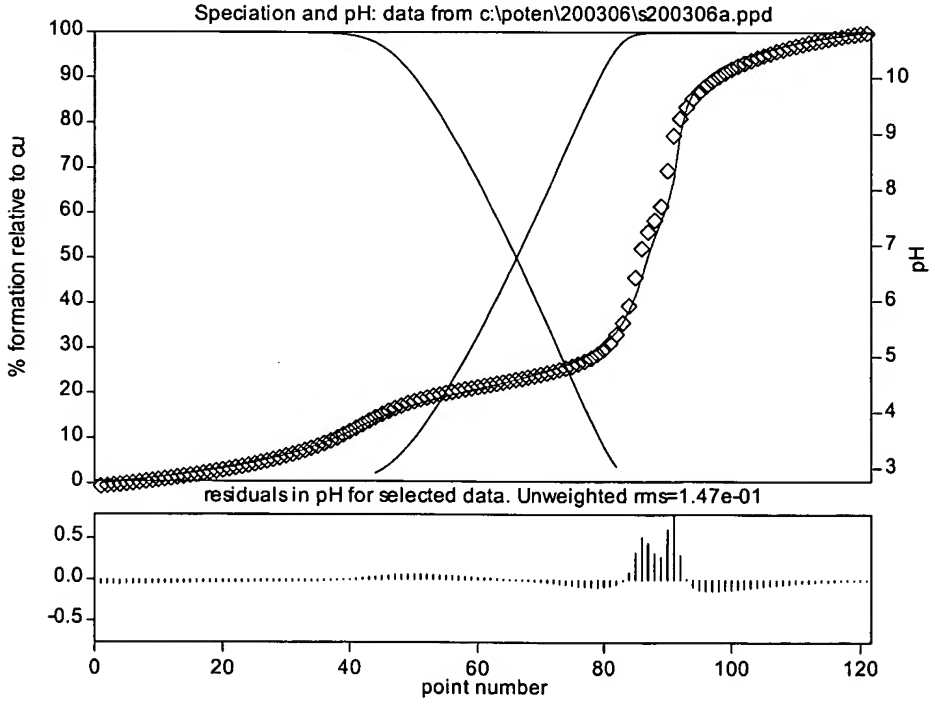
P-2 Metal Complex Stability Constant



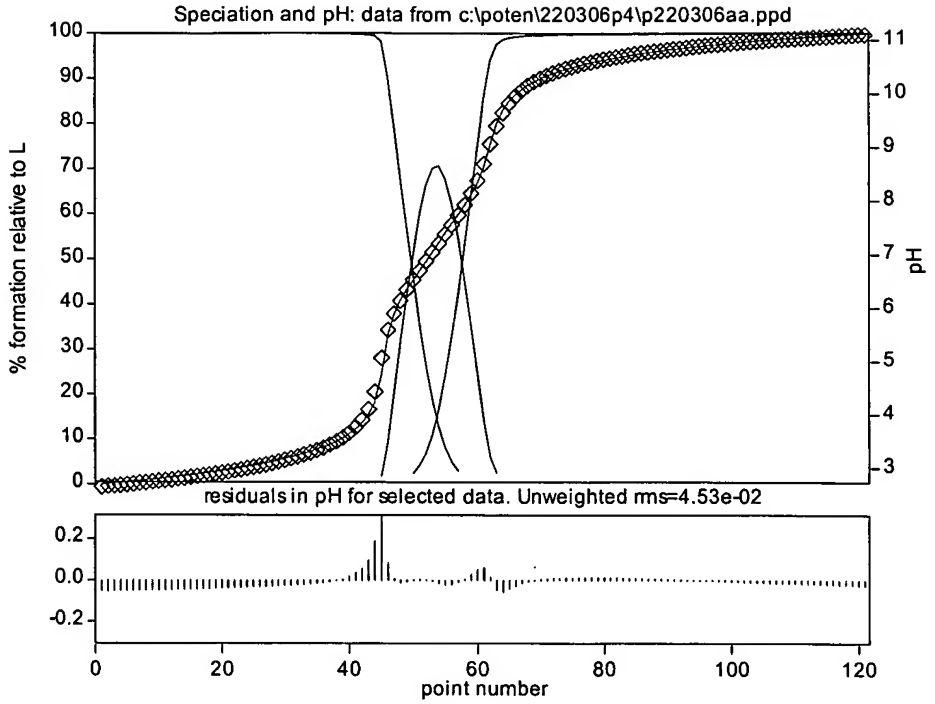
P-3 Ligand Protonation Constant



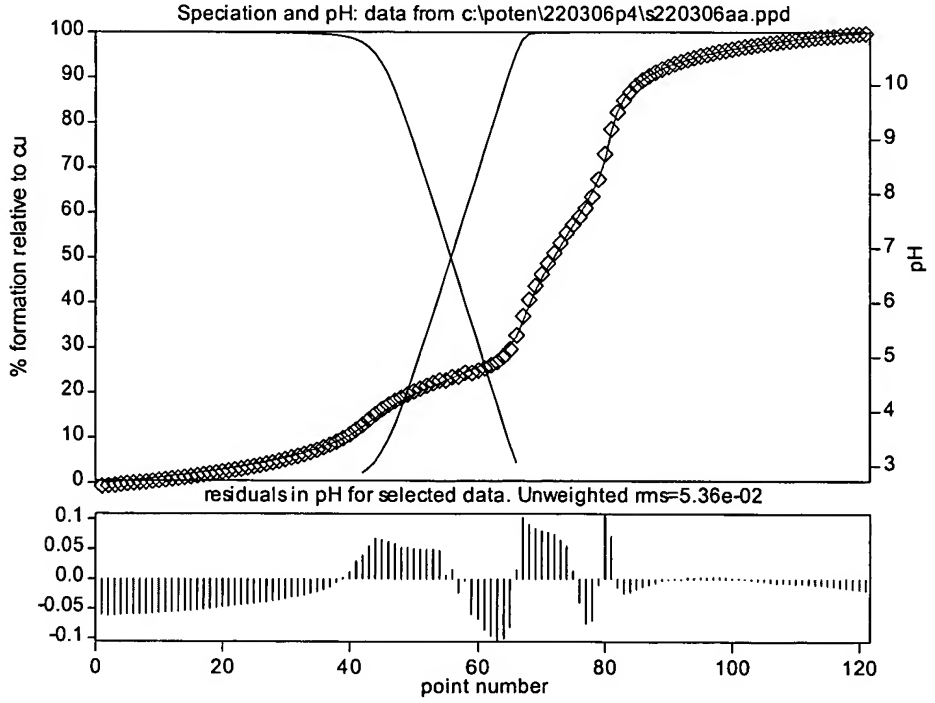
P-3 Metal complex Stability Constant



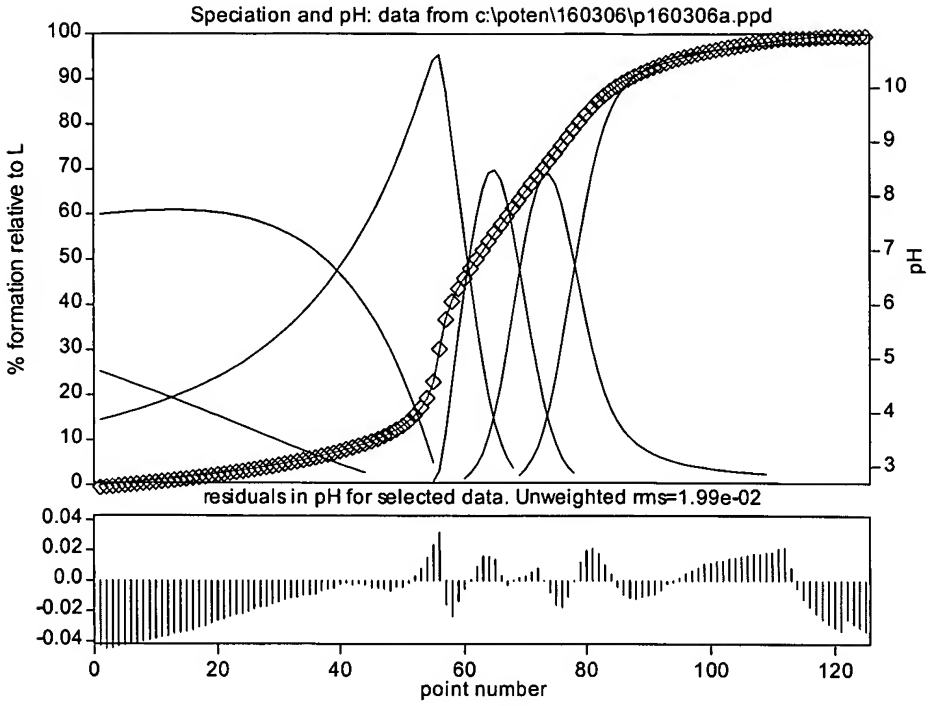
P-4 Ligand Protonation Constant



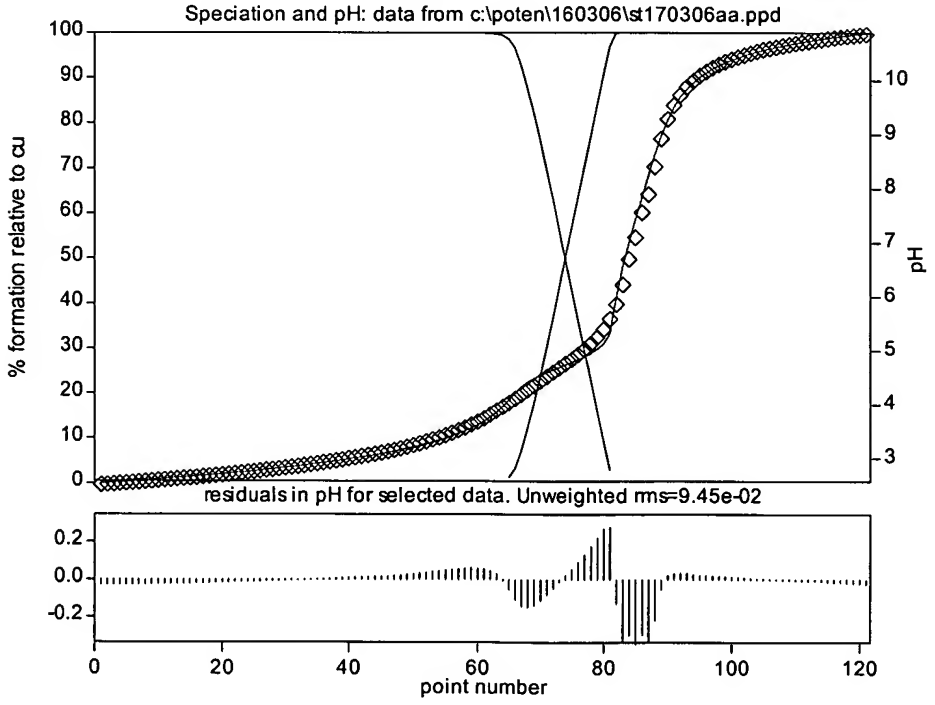
P-4 Metal Complex Stability Constant



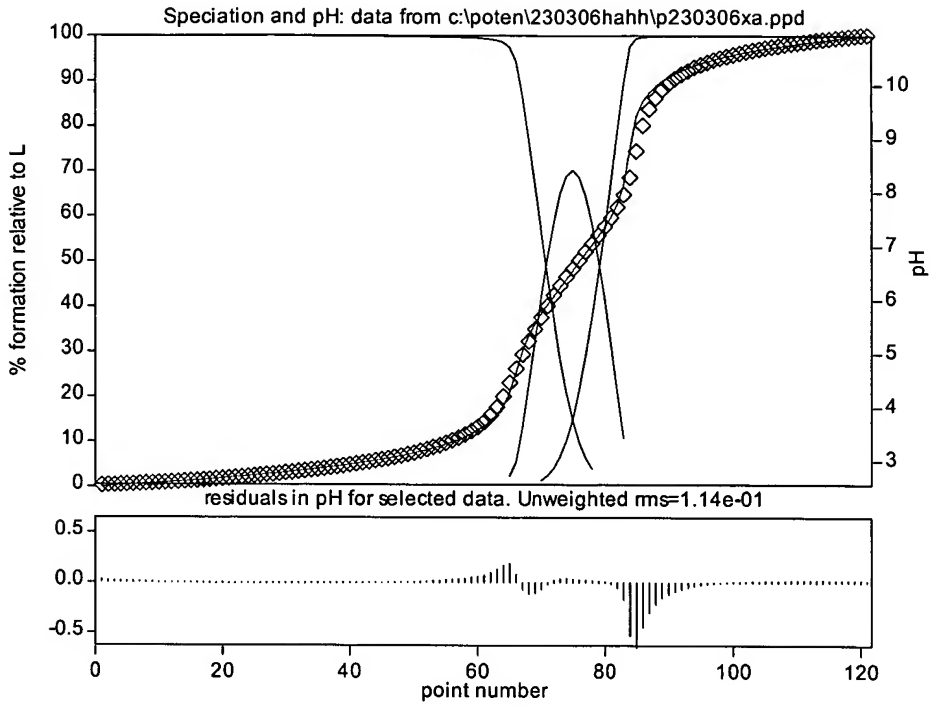
P-5 Ligand Protonation Constant



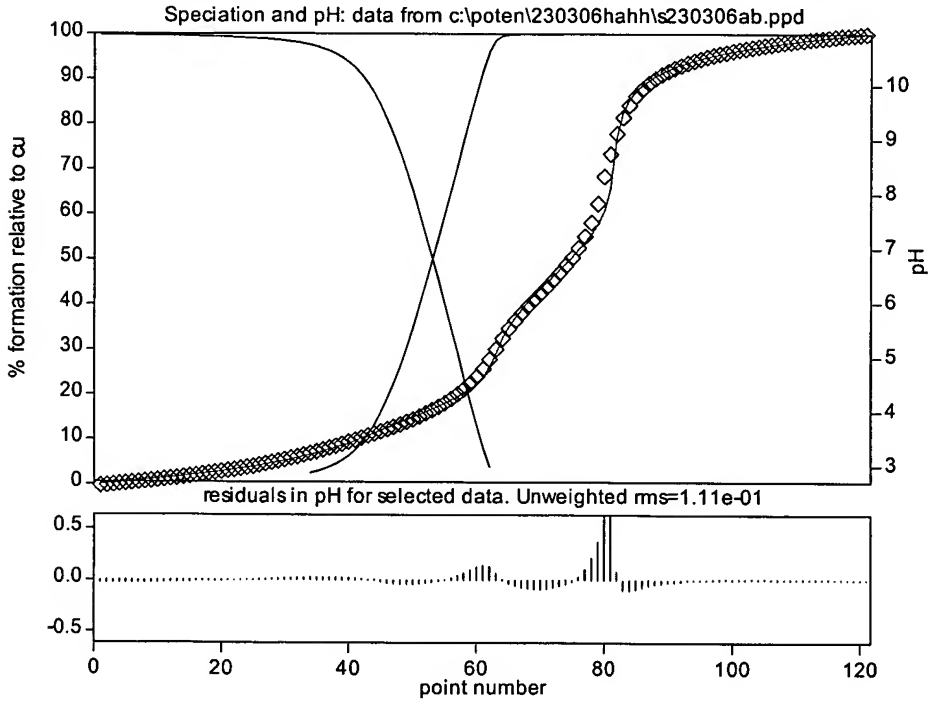
P-5 Metal Complex Stability Constant



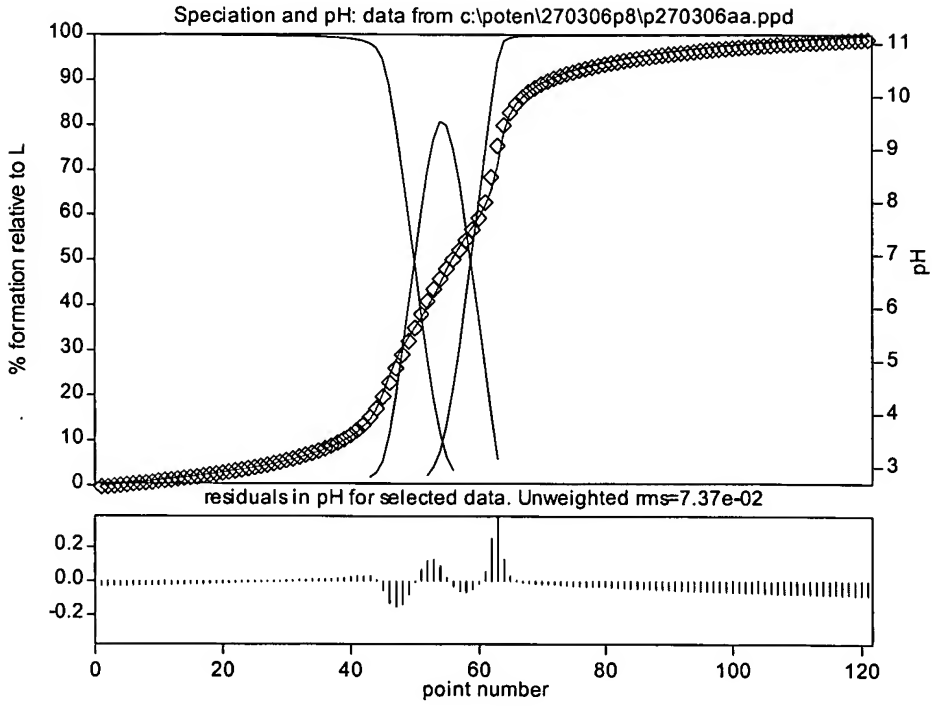
P-7 Ligand Protonation Constant



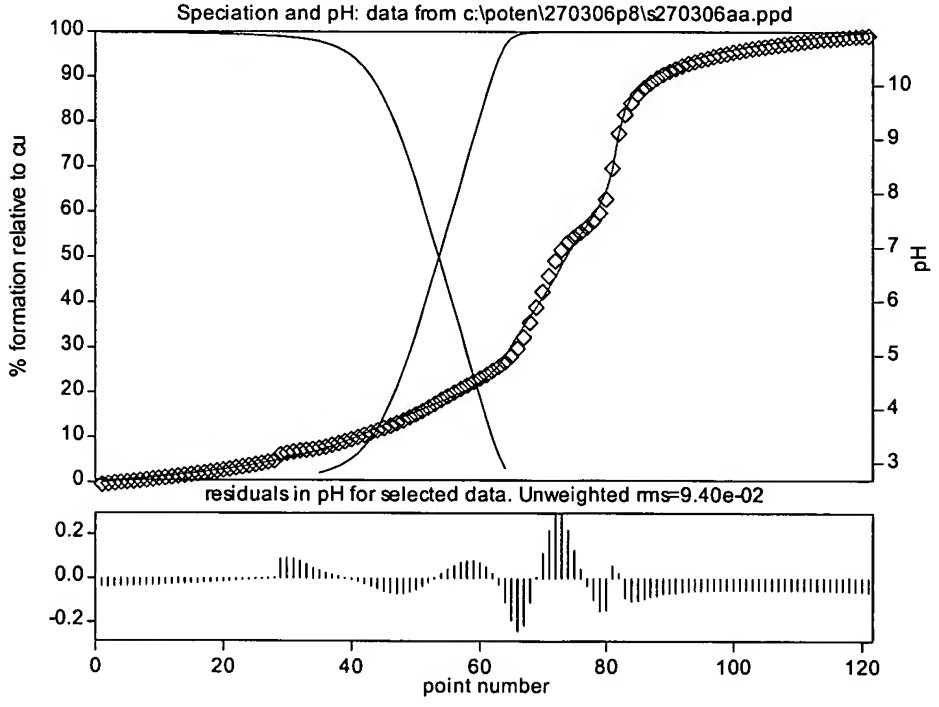
P-7 Metal Complex Stability Constant



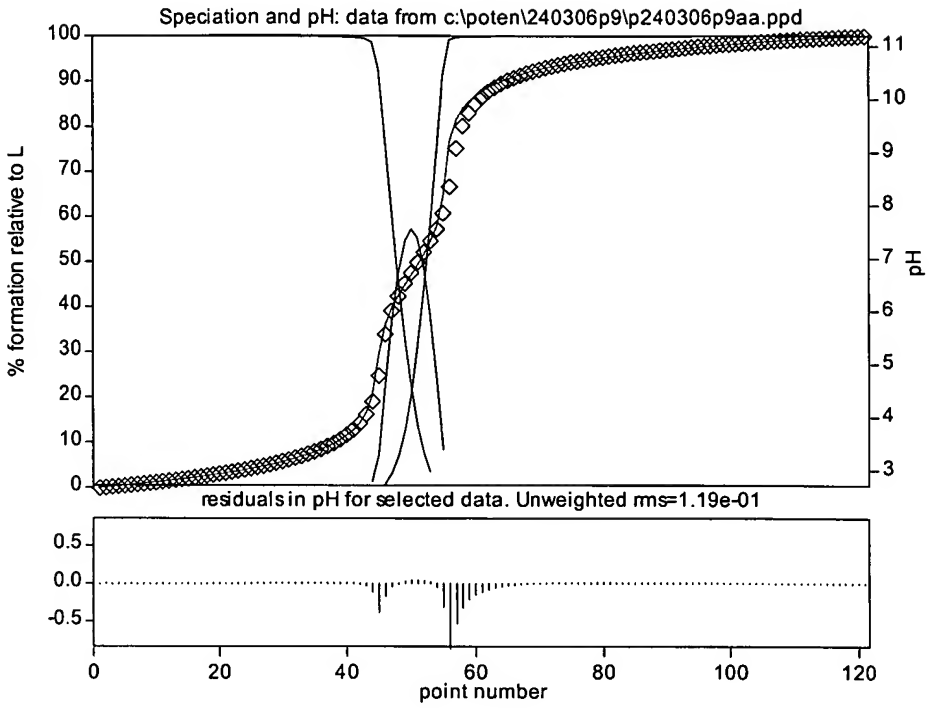
P-8 Ligand Protonation Constant



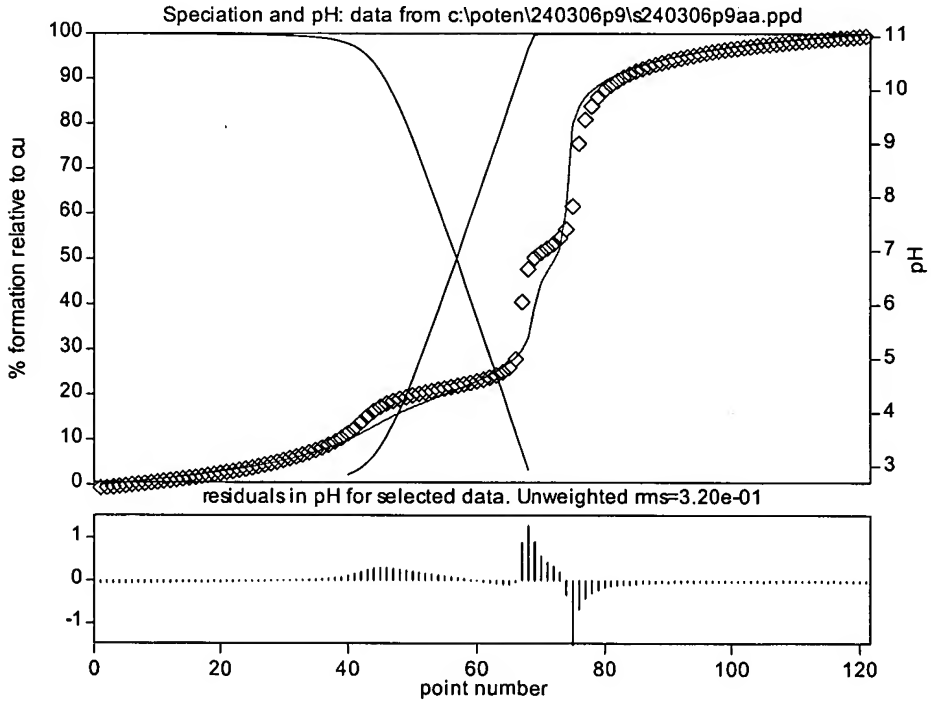
P-8 Metal Complex Stability Constant



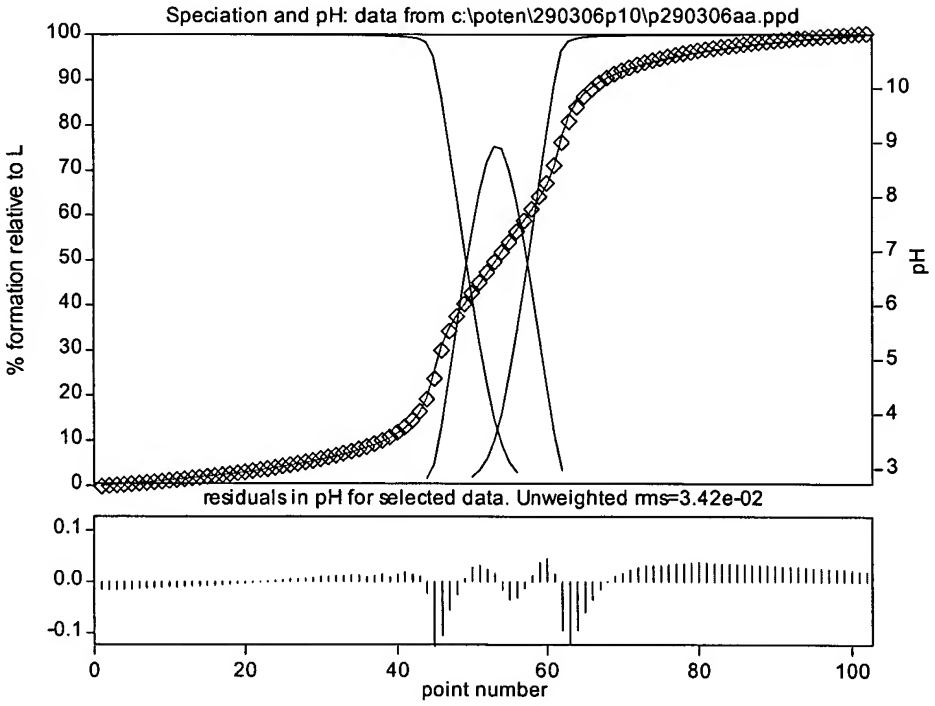
P-9 Ligand Protonation Constant



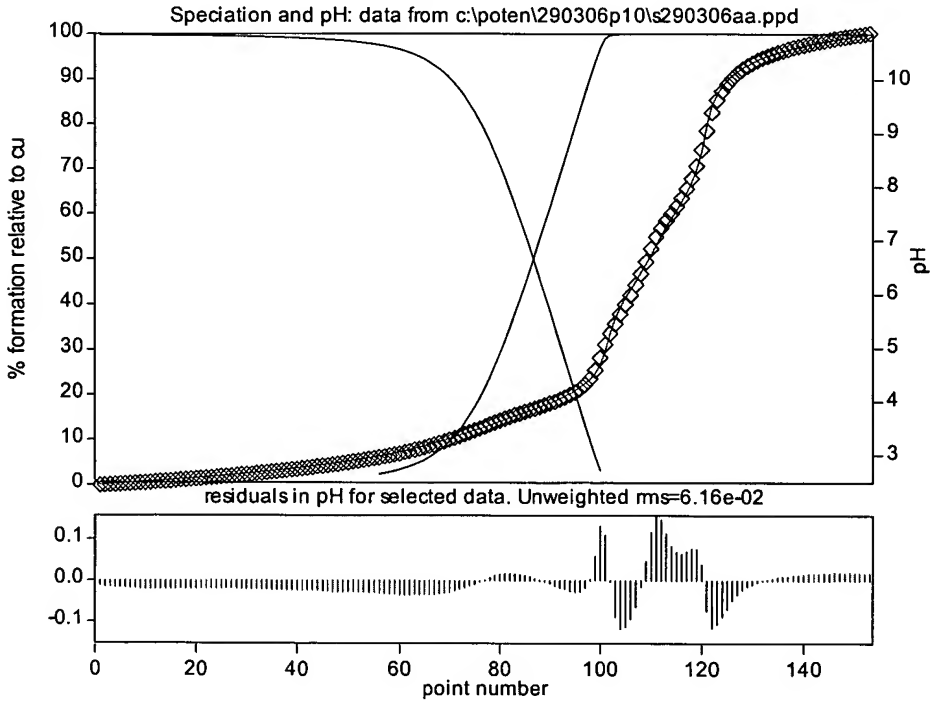
P-9 Metal Complex Stability Constant



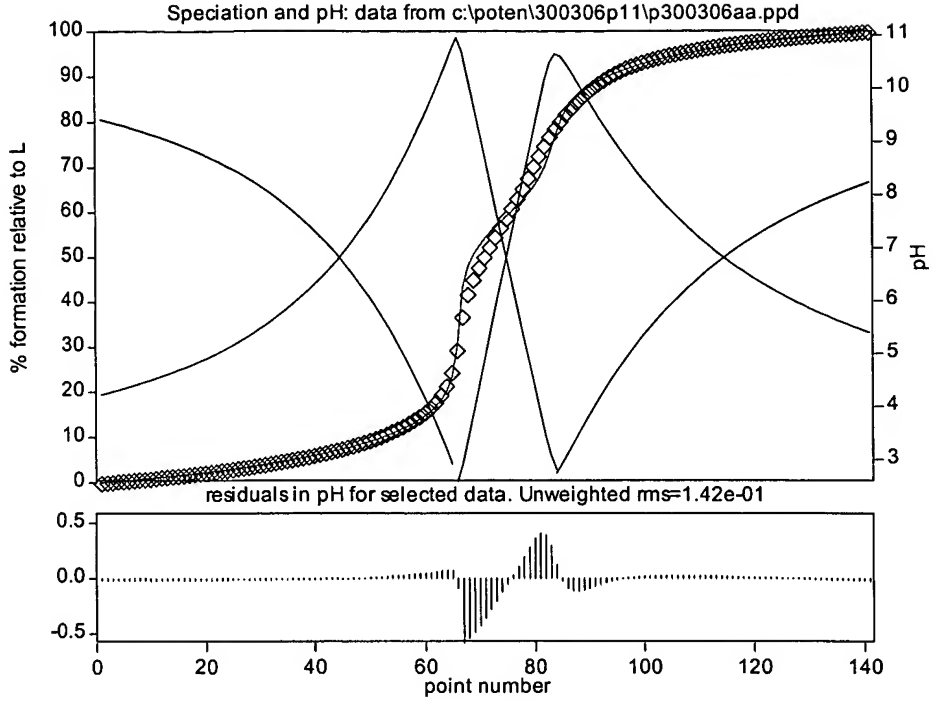
P-10 Ligand Protonation Constant



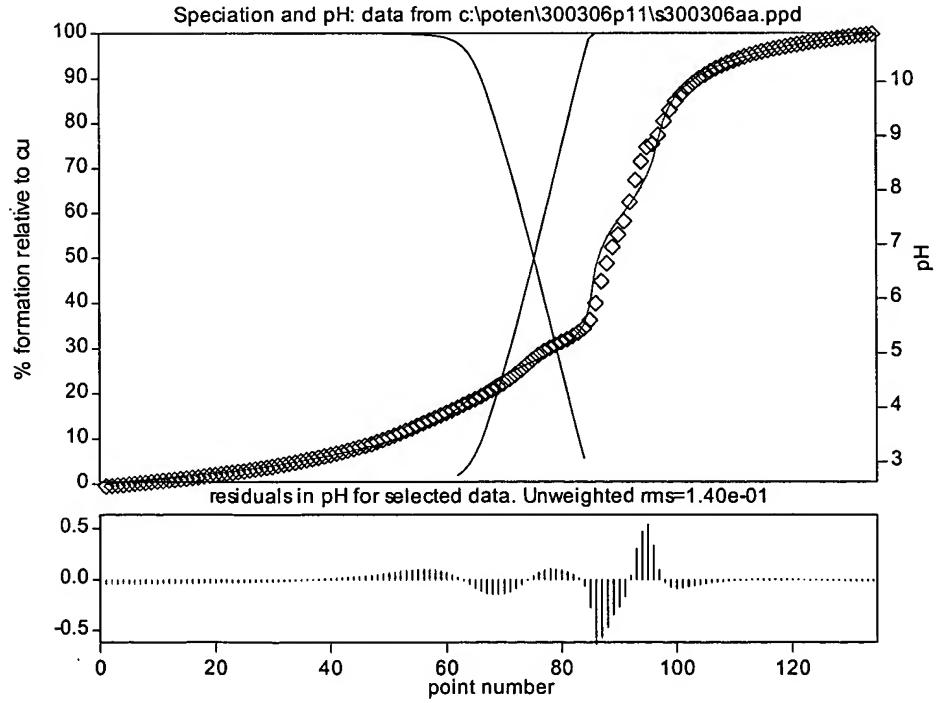
P-10 Metal Complex Stability Constant



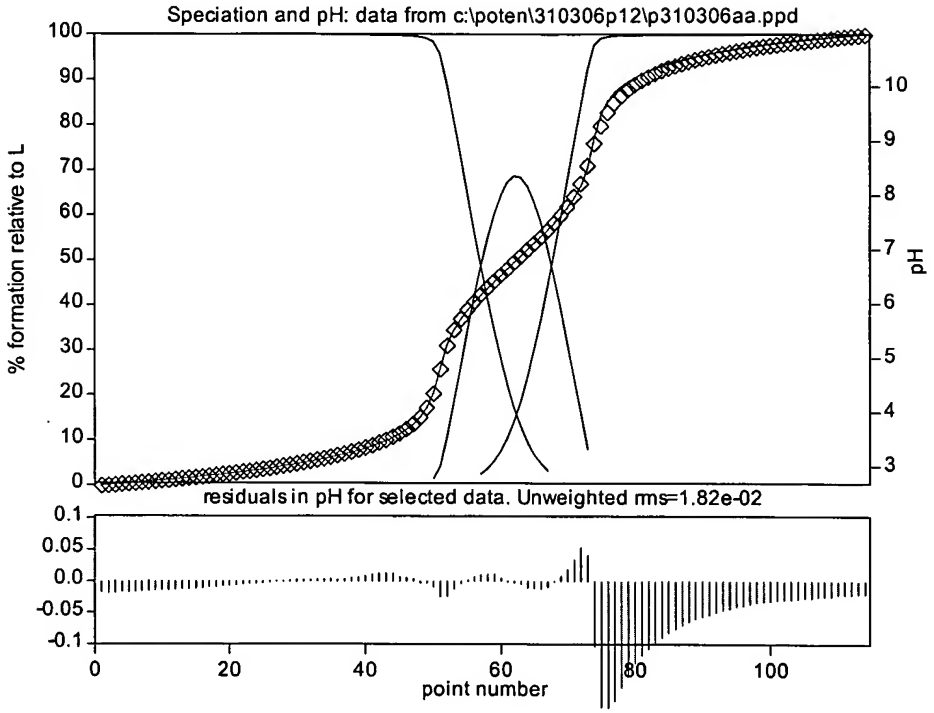
P-11 Ligand Protonation Constant



P-11 Metal Complex Stability Constant



P-12 Ligand Protonation Constant



P-12 Metal Complex Stability Constant

Not available

Chicken Chorioallantoic Membrane Assay

The peptides listed in Table A below were tested for their ability to inhibit blood vessel formation in the chicken chorioallantoic membrane assay. Stock solutions of the peptides in water were diluted with phosphate buffered saline, pH 7.4 (PBS) to give the final concentrations indicated in Table A and were sterile filtered. The solutions were coded so that the identity of the peptides was not known to those performing the assay.

Fertilized white leghorn chicken eggs (7 per group) were incubated for 3 days at 37°C and 60% humidity under continuous rotation. On the third day, a hole 2 x 1 cm was made in the eggshell of each egg. Then, the eggs were further incubated at 37°C and 60% humidity until day 10. Tape was put over the holes to prevent dehydration.

On day 10, a silicone ring was placed directly onto the chorioallantoic membrane (CAM) of each egg and was left to stabilize for 2 hours. Then, 65 μl of vehicle or test compound was placed inside the ring. This treatment was repeated daily until the end of the experiment. Each day prior to treatment, the CAMs were evaluated and screened for activity of the compounds. At day 14-17 (dependent on the amount of activity), the experiment was ended.

Photographs of the treatment area at 6X magnification were made using a Wild M8 stereomicroscope equipped with a Nikon F301 camera. The CAMs were excised and snap frozen. The vascular density indices were analyzed by digitally superimposing five concentric rings on the image of the CAM and enumeration of intersections of the rings with blood vessels. The results are presented in Table A.

TABLE A

PEPTIDE (all L-amino acids unless otherwise indicated)	**CODE**	**FINAL CONCEN-TRATION**	**NO. OF EGGS**	**VASCULAR DENSITY INDEX (mean ± standard error)**	**p value (Student t-test)**
NONE (vehicle control; 1:20 dilution of water in PBS)	___	___	5	130.8 ± 15.2	
DAHGMTCANC	A	100 μM	4	114.0 ± 19.5	0.17
DAHGMTCARC	B	100 μM	4	117.8 ± 27.7	0.38
DAHK	C	100 μM	4	92.3 ± 15.9	0.01
HAHGMTCANC	D	100 μM	4	126.3 ± 20.5	0.71
TLH	F	100 μM	4	116.3 ± 26.2	0.33
SGH	G	100 μM	4	104.8 ± 21.0	0.07
CGH	H	100 μM	4	116.0 ± 13.7	0.17
DAHKGGHH	I	100 μM	4	121.3 ± 24.7	0.50
AAHH	J	100 μM	4	94.7 ± 8.1	0.01

1: Circulation. 2000 Apr 4;101(13):1519-26.

IL-8 is an angiogenic factor in human coronary atherectomy tissue.

Simonini A, Moscucci M, Muller DW, Bates ER, Pagani FD, Burdick MD, Strieter RM.

Department of Internal Medicine, Division of Cardiology, University of Michigan Medical School, Ann Arbor, MI, USA.

BACKGROUND: Interleukin-8 (IL-8), a CXC chemokine that induces the migration and proliferation of endothelial cells and smooth muscle cells, is a potent angiogenic factor that may play a role in atherosclerosis. Previously, IL-8 has been reported in atherosclerotic lesions and circulating macrophages from patients with atherosclerosis. Therefore, we sought to determine whether IL-8 plays a role in mediating angiogenic activity in atherosclerosis. METHODS AND RESULTS: Homogenates from 16 patients undergoing directional coronary atherectomy (DCA) and control samples from the internal mammary artery (IMA) of 7 patients undergoing bypass graft surgery were assessed for IL-8 content by specific ELISA, immunohistochemistry, and in situ hybridization for IL-8 mRNA. The contribution of IL-8 to net angiogenic activity was assessed using the rat cornea micropocket assay and cultured cells. IL-8 expression was significantly elevated in DCA samples compared with IMA samples (1.71+/-0.6 versus 0.05+/-0.03 ng/mg of total protein; $P<0.01$). Positive immunolocalization of IL-8 was found exclusively in DCA tissue sections, and it correlated with the presence of factor VIII-related antigen. In situ reverse transcriptase polymerase chain reaction revealed the expression of IL-8 mRNA in DCA tissue. Corneal neovascular response, defined by ingrowth of capillary sprouts toward the implant, was markedly positive with DCA pellets, but no constitutive vessel ingrowth was seen with IMA specimens. Neutralizing IL-8 attenuated both the in vivo corneal neovascular response and the in vitro proliferation of cultured cells. CONCLUSIONS: The results suggest that, in human coronary atherosclerosis, IL-8 is an important mediator of angiogenesis and may contribute to plaque formation via its angiogenic properties.

PMID: 10747344 [PubMed - indexed for MEDLINE]

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1: J Immunol. 1997 Aug 1;159(3):1437-43.

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The CXC chemokines, IL-8 and IP-10, regulate angiogenic activity in idiopathic pulmonary fibrosis.

Keane MP, **Arenberg DA**, **Lynch JP 3rd**, **Whyte RI**, **Iannettoni MD**, **Burdick MD**, **Wilke CA**, **Morris SB**, **Glass MC**, **DiGiovine B**, **Kunkel SL**, **Strieter RM**.

Department of Internal Medicine, University of Michigan Medical School, Ann Arbor 48109, USA.

Idiopathic pulmonary fibrosis (IPF) is a chronic and often fatal disorder. Fibroplasia and deposition of extracellular matrix are dependent, in part, on angiogenesis. We postulated that an imbalance exists in the expression of angiogenic (IL-8) vs angiostatic (IFN-gamma-inducible protein (IP-10)) CXC chemokines, which favors net angiogenesis in IPF. To test this hypothesis, we obtained open lung biopsies either from normal patients undergoing thoracic surgery for reasons other than interstitial lung disease (control) or from patients with IPF. We found that levels of IL-8 were greater from tissue specimens of IPF patients then from those of controls. In contrast, IP-10 levels were higher from tissue specimens obtained from control subjects than from those from IPF patients. When IL-8 or IP-10 was depleted from IPF tissue specimens, tissue-derived angiogenic activity was markedly reduced or enhanced, respectively. Immunolocalization of IL-8 demonstrated that the pulmonary fibroblast (PF) of IPF lung was the predominant cellular source of IL-8. Isolated PF from IPF patients constitutively produced more IL-8 and less IP-10 than control PF. Conditioned media from IPF-PFs demonstrated constitutive angiogenic activity that was attributable, in part, to IL-8. Depletion of IP-10 from IPF-PF CM resulted in an increase in corneal neovascularization. These findings support the notion that IL-8 and IP-10 are important factors that regulate angiogenic activity in IPF.

PMID: 9233641 [PubMed - indexed for MEDLINE]

1: J Clin Invest. 1996 Jun 15;97(12):2792-802.

Inhibition of interleukin-8 reduces tumorigenesis of human non-small cell lung cancer in SCID mice.

Arenberg DA, Kunkel SL, Polverini PJ, Glass M, Burdick MD, Strieter RM.

Department of Internal Medicine (Division of Pulmonary and Critical Medicine), University of Michigan Medical School, Ann Arbor 48109, USA.

The salient feature of solid tumor growth is the strict dependence on local angiogenesis. We have previously demonstrated that IL-8 is an angiogenic factor present in freshly isolated specimens of human non-small cell lung cancer (NSCLC). Using a model of human NSCLC tumorigenesis in SCID mice, we now report that IL-8 acts as a promoter of human NSCLC tumor growth through its angiogenic properties. Passive immunization with neutralizing antibodies to IL-8 resulted in more than 40% reduction in tumor size and was associated with a decline in tumor-associated vascular density and angiogenic activity. IL-8 did not act as an autocrine growth factor for NSCLC proliferation. The reduction in primary tumor size in response to neutralizing antibodies to IL-8 was also accompanied by a trend toward a decrease in spontaneous metastasis to the lung. These data support the notion that IL-8 plays a significant role in mediating angiogenic activity during tumorigenesis of human NSCLC, thereby offering a potential target for immunotherapy against solid tumors.

PMID: 8675690 [PubMed - indexed for MEDLINE]

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1: J Exp Med. 1994 May 1;179(5):1409-15.

Related Articles, Links

Inhibition of interleukin 8 attenuates angiogenesis in bronchogenic carcinoma.

Smith DR, Polverini PJ, Kunkel SL, Orringer MB, Whyte RI, Burdick MD, Wilke CA, Strieter RM.

Department of Internal Medicine, University of Michigan Medical School, Ann Arbor 48109-0360.

We investigated the role of interleukin 8 (IL-8) in mediating angiogenesis in human bronchogenic carcinoma. Increased quantities of IL-8 were detected in tumor tissue as compared with normal lung tissue. Immunohistochemical staining of tumors revealed primary localization of IL-8 to individual tumor cells and demonstrated the capacity of tumor to elaborate IL-8. Functional studies that used tissue homogenates of tumors demonstrated the induction of both in vitro endothelial cell chemotaxis and in vivo corneal neovascularization. It is important to note that the addition of neutralizing antisera to IL-8 to these assays resulted in the marked and specific attenuation of these responses. Our observations definitively establish IL-8 as a primary mediator of angiogenesis in bronchogenic carcinoma and offer a potential target for immunotherapies against solid malignancies.

PMID: 7513008 [PubMed - indexed for MEDLINE]

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1: Am J Pathol. 1992 Dec;141(6):1279-84. Related Articles, Links

Interleukin-8. A corneal factor that induces neovascularization.

Strieter RM, Kunkel SL, Elner VM, Martonyi CL, Koch AE, Polverini PJ, Elner SG.

Department of Internal Medicine, University of Michigan, Ann Arbor.

A rabbit corneal pocket model was used to demonstrate that physiologic concentrations of human recombinant (r) IL-8 may induce corneal neovascularization. Computer-assisted analysis of sequential fluorescein angiograms showed that rIL-8 doses ranging from 2 to 40 ng/cornea ($P = 0.01$), but not high dose rIL-8 (400 ng/cornea), results in neovascularization within 14 days. Repeat fluorescein angiograms 6 weeks after placing angiogenic doses of rIL-8 demonstrated significant regression ($P = 0.01$) of the vascularity present at 2 weeks, suggesting that IL-8 angiogenesis undergoes dynamic modulation similar to that normally seen in wound healing. To our knowledge, this is the first study showing an angiogenic role for IL-8, a finding that emphasizes the interplay between inflammation and wound healing. Our results imply that corneal-derived IL-8 may be important in corneal neovascularization, in particular, and that IL-8 may modulate wound healing in general. Finally, these results raise the possibility that corneal-derived cytokines, such as IL-8, may obfuscate the effects of agents tested in experimental corneal pocket models.

PMID: 1281615 [PubMed - indexed for MEDLINE]